THE MORPHOLOGY OF POST-TRAUMATIC OSTEOPENIA

By

KARL J. OBRANT

Malmö 1984

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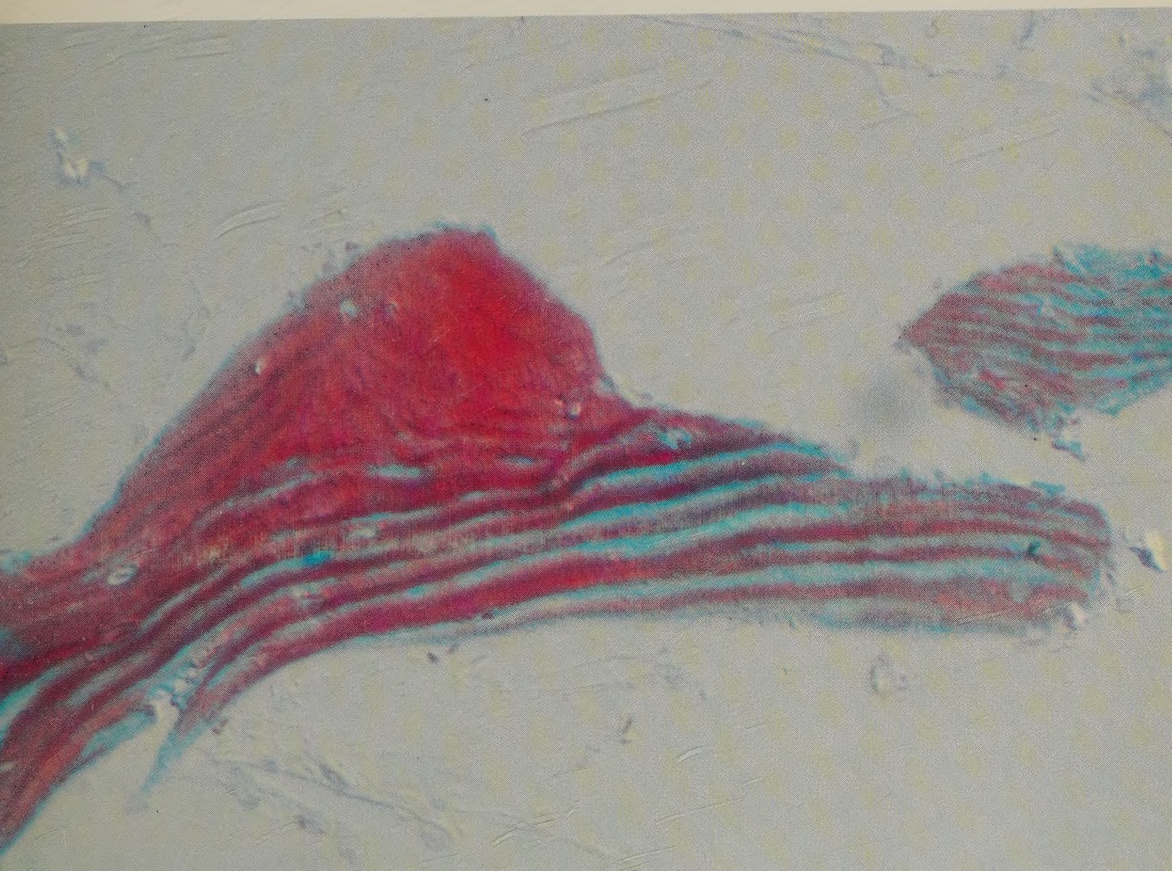

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Abstract

With the objective of studying the morphology of post-traumatic osteopenia, bone samples were secured from the affected bone in 24 coxarthrosis patients in conjunction with total hip replacement, from 32 femoral neck fracture patients in conjunction with secondary fracture surgery and from 20 patients with tibia shaft fractures in conjunction with stabilizing operations in delayed union. Also, from 42 cadavers altogether 168 samples from the trochanteric region and from the upper end of the tibia were collected as reference material.

In the fracture patients there was an increased abundance of osteoid tissue and of osteoclasts. There was a pattern of sandwiched structures of osteoid tissue and mineralized bone - in some instances entirely unmineralized trabeculae.

The enlarged surfaces of osteoid tissue on the trabeculae were covered with osteoblast cells in the same proportion as normal - with the increased osteoid tissue abundance, however, the osteoblasts were increased in number.

With the quantitative electron microscopy technique the calcium content was measured in a series of samples with changes typical of post-traumatic osteopenia. It was discovered that even if the colour changes, in histochemical preparations designed to measure osteoid, were sharp, the actual mineralization changed gradually through the trabeculae.

With quantitative measurements of small areas located centrally in the trabeculae it was found that in trabeculae from patients with post-traumatic osteopenia the calcium content could vary from normal to a reduction by 100%.

An additional study included the roentgen examination of the femora of patients who had,in the past,sustained a tibia shaft fracture.There was a thinning of the cortices,resulting in a slight widening of the marrow cavity which did not change in time. Post-traumatic osteopenia is, from the morphological point of view,a high turn-over condition of several years' duration,which finally reaches a steady state usually with a small net loss of bone.

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Akademisk avhandling som med vederbörligt tillstånd av Medicinska Fakulteten vid Universitetet i Lund för erhållande av medicine doktorsexamen kommer att offentligen försvaras å Medicinska klinikens föreläsningssal, Allmänna Sjukhuset i Malmö, fredagen den 13 april 1984 kl. 14.00.

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This survey is based on the following publications:

I. Trabecular bone changes in the greater trochanter after fracture of the femoral neck.
Karl J. Obrant. Acta Orthop Scand 55:78-82, 1984.

II. Histomorphologic changes in the tibial epiphysis after diaphyseal fracture.
Karl J. Obrant & Bo E. Nilsson. Clin Orthop 185:148-153, 1984.

III. Electron microprobe analysis and histochemical examination of the calcium distribution in bone trabeculae in man.
A methodological study, using biopsy specimens from post-traumatic osteopenia.
Karl J. Obrant & Rolf Odselius. Submitted for publ.

IV. Electron microprobe investigation of the concentration of calcium and phosphorus in bone trabeculae in man - both normal and in post-traumatic osteopenia.
Karl J. Obrant & Rolf Odselius. Submitted for publ.

V. Post-fracture changes of the femur cortex.
Bo E. Nilsson & Karl J. Obrant. Acta Orthop Scand 54:862-864, 1983.

These articles are referred to in the text by the Roman numerals given above.

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INTRODUCTION

Already in 1862 Volkmann described local changes in bone after trauma. In the early roentgen era Sudeck (1900) described changes following immobilization. Sudeck thought at the time that an inflammatory disease was necessary for such local bone atrophy. Kienböck (1901) described the roentgenological changes in more detail - cortical thinning and rarefication of the trabeculae. Exner (1902) reported cases in which trauma and immobilization were the only causes of bone atrophy. Herfarth (1924) described 33 cases with bone atrophy after fracture. Basing his opinion on 20 cases with soft tissue injuries he proposed that even a mild trauma could be the cause of such atrophy. In some of his cases the atrophy was visible in roentgenograms already three weeks after the trauma. The early literature on post-traumatic osteopenia has been reviewed by Beck (1925).

Roentgen and radiometry

Lacroix & Ponlot (1956) found that the post-traumatic bone loss first occurs in the subchondral bone and is mainly localized to the metaphysis. Belenger (1956) and Ronse (1963) reported roentgenological healing of osteopenia within a year after injury. On the other hand, Steinbach (1964) pointed out that roentgenological signs of post-traumatic osteopenia may be visible up to nine years after fracture.

Gamma-absorptiometry has become a useful tool for investigating post-traumatic osteopenia. By this method Nilsson (1966) found that the mineral content of a fractured limb was almost always decreased even when more than a decade had elapsed after the injury - the bone mineral mass decreased not only in the fractured bone but also in other bones of the same limb. In patients who had had additional trauma from secondary surgery,the bone mineral loss was even greater. Nilsson (1966), Westlin (1974) and Andersson & Nilsson (1979a) found a tendency to restore mineral after trauma. Participation in sports and physical activity may increase bone mineral mass (Nilsson & Westlin 1971, Dalén & Olsson 1974, Andersson 1978). On the other hand, after fracture, functional

bracing and weight bearing did not influence the rate of bone mineral loss (Andersson & Nilsson 1979b).

Kinetic and chemical studies

Bone-seeking radio-nuclides have been used for the purpose of studying post-traumatic osteopenia (Bohr & Sørensen 1950, Bauer & Carlsson 1955, Wendeberg 1961). Wendeberg found a high uptake of calcium tracers in the femur of patients with a fractured ipsilateral tibia. This implies an increased bone formation which, in conjunction with the mineral loss found by quantitative in vivo measurements, suggests that post-traumatic osteopenia is a high turn-over condition. Similar observations were made by Kharmosh & Saville (1965) in rabbits paralyzed by nerve resection. After external immobilization of animals Eichler (1970) and Mattsson (1972) found decreased ash weights. Wredmark (1977) had similar results after fracture and immobilization. Landry & Fleisch (1964) measured the absolute amount of tetracycline accumulated in immobilized rat legs. They found that after a short initial phase, during which bone formation was reduced, it increased and remained high for a long time and then, finally, became subnormal as compared with the contra-lateral leg. The bone weight decreased continuously during the high uptake period. The authors concluded that the loss of bone mineral was due to an increased rate of resorption.

Morphology

Cellular components

Rieder (1936) found an increased number of osteoclasts in human bone tissue after trauma and could confirm his findings in animal experiments. Also, Geiser & Trueta (1958) found increased numbers of osteoclasts in fractured bones in rabbits, but also osteoblast counts were increased. Uhthoff & Jaworski (1978) reported increased amounts of resorptive surfaces in immobilized dogs, a histomorphometric parameter often used to describe the osteoclast activity. Also, in immobilized rabbits Kühr (1982) found an increased width of the osteoblast cell layer covering osteoid tissue.

Mineralization

In recent decades bone histomorphology has been augmented by the possibility of examining undecalcified sections. The preparation technique has been described by Arnold & Jee (1954), Baron et al. (1983) and others. The histomorphometric analysis method described by Frost (1969) and Merz & Schenk (1970) together with various staining methods (Frost 1959) has made it possible to quantify the amount of osteoid in an undecalcified section of bone.

There is no consensus on the composition of bone influenced by trauma - whether or not there is incomplete mineralization or whether indeed all components of bone mineral and matrix are reduced. Landoff (1942) found in rabbits after trauma no change in the amount of osteoid. Minaire et al. (1974) reported that in iliac crest biopsies from immobilized patients, osteoporosis became worse until the 25th week after which a steady state was attained. The amount of osteoid tissue was somewhat decreased. Uhthoff & Jaworski (1978) found unchanged amounts of osteoid seams in immobilized animals.

However, most investigators of post-traumatic and immobilization osteopenia have found an increased amount of osteoid tissue. Rieder (1936) reported more than normal abundance of osteoid tissue after trauma both in man and rabbit - in man the change occurred already after two to three weeks. Similarly, Freudiger (1950) found increased amounts of unmineralized bone in the bones of rabbits after fracture. However, the change became apparent first after several months - poor fracture alignment caused more severe changes. Geiser & Trueta (1958), who proposed that immobilization rather than fracture was important for the post-traumatic bone changes, could demonstrate an increased volume of unmineralized bone in their rabbit studies. By quantitative micro-radiography in two cases Baud & Pouezat (1973) found that post-traumatic osteopenia in man was associated with low mineralization of the bone trabeculae.

Using the electron diffraction technique, Frank et al (1957) found in one case mineral crystals smaller than normal in bone after

trauma. This has been confirmed in immobilized monkeys in recent years by Grynpas (1983). By means of the electron probe technique, Bunch et al.(1982) have shown that there is a lowered mineral content in cortical bone in immobilized monkeys. Quantitative electron microscopy has, so far, not been applied in studies of post-traumatic bone changes in man.

The aim of this investigation was:

To obtain suitable samples of trabecular bone from patients suspected to have post-traumatic bone changes.

To describe changes in the osteoclast - osteoblast balance associated with trauma.

To evaluate the degree of mineralization of trabecular bone in fractured limbs using histomorphometric techniques.

To evaluate these same techniques by comparing the results with data from electron probe measurements of bone trabeculae from fracture patients.

To find out whether the previously described, long-lasting loss demonstrated in trabecular bone after fracture can occur also in the human bone cortex.

DISCUSSION

The biopsy site

Bone biopsy samples from healthy individuals are not easily obtained - nevertheless, normative data from the iliac crest have been set up by Melsen et al.(1978). When it comes to biopsies on patients, the ethical problems are almost insurmountable. Biopsy specimens suitable for morphometric evaluation must be of a considerable size and may have to be taken from already injured tissues.

A first attempt was made to obtain material from necropsies of individuals who had, for a suitable time, survived an accident with one or more fractures. These proved to be such rare events that one could not hope to obtain a reasonable amount of data within a reasonable period of time.

Another possibility was provided by patients who had sustained fracture and were to be reoperated for the purpose of dealing with fracture complications or for surgical procedures to promote bone union. The selection of the greater trochanter (I) and the upper end of the tibia (II, III, IV) for biopsies was therefore not dictated by choice but by necessity. Pieces of bone which had to be removed in any case, could be obtained from these sites without any disadvantage whatsoever to the patient.

Given this choice, the tibia biopsy site is nevertheless an interesting area since we know from past experience that the proximal end of the tibia participates in the metabolic changes caused by a fracture (Wendeberg 1961, Andersson & Nilsson 1979a), Also, this site was always at such a distance from the fracture itself that the fracture healing - the callus formation - could not have interfered with the biopsy bone.

This may not be the case in the trabecular bone removed from the greater trochanter in patients with femoral neck fracture. Nevertheless, the changes observed after fracture were more obvious in the tibia than in the trochanter in spite of the proximity to the

fracture of the latter site.

The histological appearance of post-traumatic osteopenia

There was a vast increase of osteoid tissue in trabeculae from fractured bones, with a tendency of the osteoid tissue to appear in layers sandwiched between mineralized bone. This phenomenon was found in both biopsy sites (I, II) but was much more pronounced in the tibia (front cover).But for a few exceptions (Raina 1973) the existence of osteoid within mineralized bone is not discussed in the literature - it may occasionally be observed in renal osteomalacia (Johnell 1984). Sandwiched osteoid tissue throughout entire trabeculae has not been described before and is probably specific for post-traumatic osteopenia. The uneven distribution of osteoid tissue between and within trabeculae in the same section is also typical of this condition. Singular trabeculae may stand out as completely osteoid but surrounded by otherwise mineralized bone tissue, whereas in some specimens almost all the trabeculae were composed of osteoid tissue. The ratio osteoid volume/osteoid surfaces is increased in post-traumatic osteopenia. However, the osteoid seam width index is not an appropriate parameter for describing the morphology of this condition, since true osteoid seams were scarse as compared with the total amount of osteoid.

The biopsy technique and the Goldner staining procedure have been used in our laboratory for several years (Johnell et al.1977). The estimation of osteoid parameters is probably very sensitive to minor variations in the technique - normative data on osteoid volume in the iliac crest vary by a factor of 20 (Wiklund 1980).

Furthermore, there is electron microscopic evidence in this study that the characteristic appearance of osteoid is not a staining artefact (III, IV). The border line between the osteoid tissue and the mineralized bone,as it appears after the various staining techniques, is only due to a colour change at a certain concentration of calcium in the gradual change of calcium content, at least in post-traumatic osteopenia. If this is so, and our data support this concept, it may explain the differences in normative osteoid data (Figure 1).

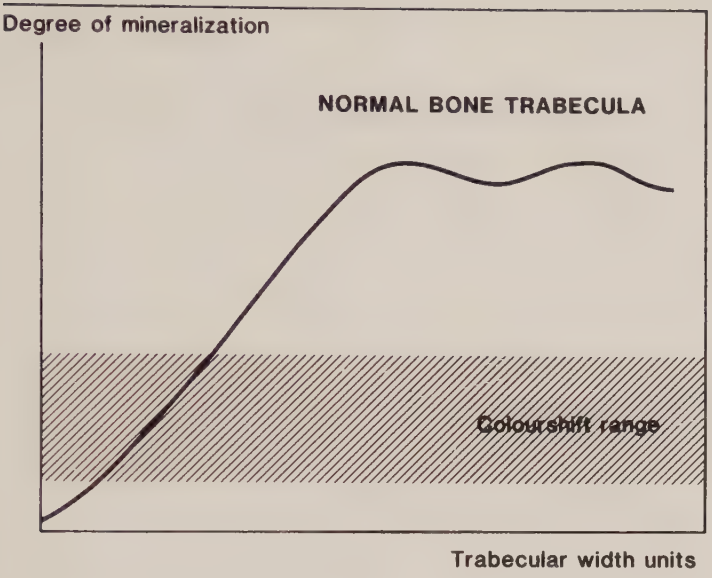

Figure 1. Hypothesis of the relationship between mineralization and staining of osteoid tissue.
Methodological variations influencing the relationship between colour change and mineralization may considerably modify the outcome with regard to the abundance of osteoid tissue.

Meyer (1960) found calcium crystals deposited in bone in a striated pattern and further it is known that there is a rhythmic pattern in the mineralization of osteons (Sissons 1962).If, in a bone trabecula which has undergone post-traumatic alteration, the concentration of calcium is below normal, the striation phenomenon in the sections is explained (Figure 2). Also, if the rate of the turn-over of bone - formation and resorption - were erratic and in general increased in the course of the metabolic response to bone injury and fracture healing, islands of bone or entire trabeculae with low mineralization could appear in a haphazard manner.

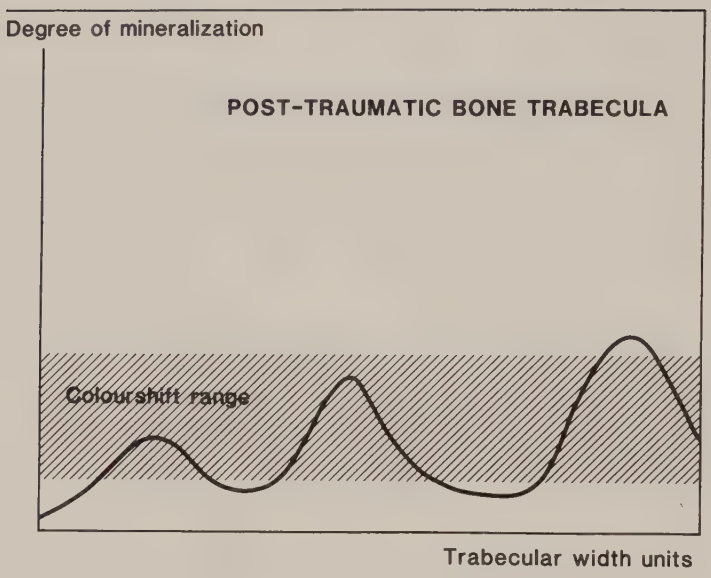

Figure 2. Hypothesis of the relationship between mineralization and staining of osteoid tissue.
Depending on the degree of mineralization required for a colour change the appearance of a section may range from osteoid tissue with layers of bone to bone with layers of osteoid tissue.

Post-traumatic osteopenia - a high turn-over condition?

Already in 1857 Kilian described one possible way of bone resorption - "halisteresis". Jaffe (1930) suggested two additional mechanisms for bone resorption - vascular resorption and osteoclastic resorption. Today, only few would disagree with the hypothesis of osteoclastic resorption, which is possibly the only way in which bone is resorbed. However, there are those who believe in osteocytic bone resorption (Bélanger 1969). According to Johnell (1980) the osteoclast count is probably the best parameter for the evaluation of bone resorption. Our data show clearly an increased osteoclast count in bone having undergone post-traumatic alteration (I, II).

The ratio active/inactive osteoid tissue, as judged from the presence of active osteoblasts, was the same in post-traumatic osteopenia as in normal trabecular bone, but the vast increase of osteoid tissue, including the active surfaces (I, II),implies a high rate of bone formation. The same conclusion was reached by Wendeberg (1961) using radionuclide tracers, Landry & Fleish (1964) in chemical studies and Obrant (1984) by tetracycline double labelling.The abundance of osteoid tissue in post-traumatic osteopenia is probably the result of defective mineralization and not a demineralization of existing bone. Perhaps the apposition rate is too high for the calcium to be deposited in normal amounts. Earlier investigations of calcification (Rosenberg & Simmons 1979) have shown a circadian rhythm in the mineralization of animal dentin. This might be an explanation of the sandwiching of osteoid tissue in post-traumatic osteopenia and the fact that some electron probe measuring sites in trabecular bone have a normal calcium content and some a reduction by almost 100% (III, IV).

The time sequence of post-traumatic osteopenia in man

The loss of bone mineral, often as much as 50% during the first months after fracture (Westlin 1974, Andersson & Nilsson 1979a) is due to the fact that resorption exceeds formation. Osteoclasts are more abundant during the first year after fracture (II). From earlier studies it is also apparent that post-traumatic osteopenia is in part a reversible condition (Nilsson 1966, Andersson & Nilsson (1979a). It seems plausible that this reversion comes after a change in cellular activity so that the osteoblast activity exceeds the osteoclast activity.

From this study it would also appear that the osteoid volume reaches its peak value at the time when the change in cellular activity sets in (II). In Figure 3 a time sequence hypothesis is presented for post-traumatic osteopenia in man. On this hypothesis the reversibility of post-traumatic osteopenia is seen not as a restoration of bone volume but of bone mineral. In addition, there is some evidence in favour of a small initial loss of bone volume which may not be reversible.

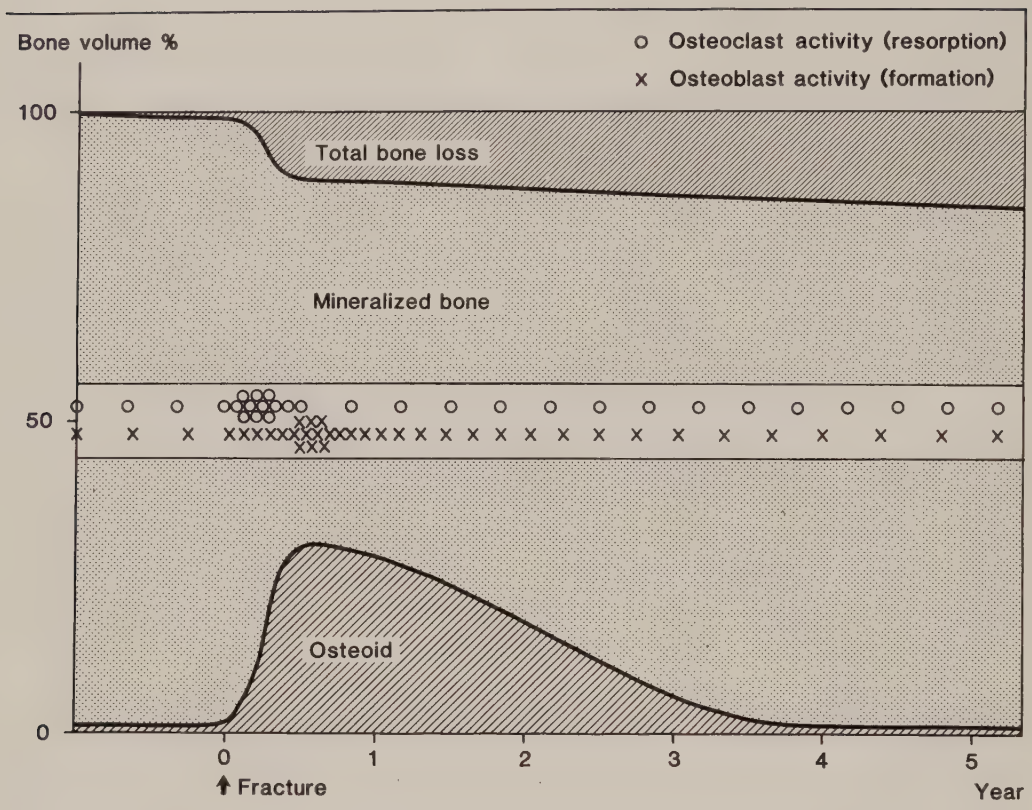

Figure 3. Hypothesis of the sequence of events in post-traumatic osteopenia. When the fracture occurs there is a rapid loss of bone which is almost totally replaced by osteoid tissue laid down in the course of the rapid repair process. The net effect is a considerable decrease in the amount of mineralized bone. After a few years this repair process slowly subsides with a net loss of bone as the final result. Response by the cellular elements is a rapidly increased osteoclast activity followed within a short time by a high osteoblast activity. In time these cellular components normalize.

Changes in coxarthrosis

Also coxarthrosis is a high turn-over condition. Danielsson et al. (1964) found an increased uptake of bone-seeking radionuclide tracers in coxarthrosis at various stages of severity. Bach Christensen & Arnoldi (1980) found, in femoral heads removed in conjunction with total hip replacement, an accumulation of tracer (Tc-phosphorus compound) in areas of new-bone formation. Patients selected for hip replacement, such as those included in the present study (I), have advanced disease - possibly with a repair reaction involving the entire upper end of the femur. In the study of bone morphology in the upper end of the femur the changes were more obvious in patients with femoral neck fractures than in those with coxarthrosis, but the latter group was clearly disqualified as a standard of normality.

The steady state?

Will the metabolic turmoil triggered off by a fracture ever be terminated so that a steady state is again attained? In previous studies the answer to this is not quite clear (Wendeberg 1961, Nilsson 1966, Westlin 1974, Andersson 1978). Even if, after a few years, the high turn-over rate decreases, some lost bone is still being recovered.

The femur participates in the metabolic changes - the high turn-over - which occur after a tibia fracture (Wendeberg 1961). However, after a few years there seems to be no further change in the bone mass as measured from radiograms, nor is the bone formation increased (Wendeberg 1961). A steady state is attained, again with a net loss as a result of the fracture. In a roentgenogram the result is a slight widening of the marrow cavity which remains unchanged in time (V).

SUMMARY AND CONCLUSIONS

With the objective of studying the morphology of post-traumatic osteopenia, bone samples were secured from the affected bone in 24 coxarthrosis patients in conjunction with total hip replacement, from 32 femoral neck fracture patients in conjunction with secondary fracture surgery and from 20 patients with tibia shaft fractures in conjunction with stabilizing operations in delayed union. Also, from 42 cadavers altogether 168 samples from the trochanteric region and from the upper end of the tibia were collected as reference material.

In the fracture patients - femoral neck and tibia shaft - there was an increased abundance of osteoid tissue and of osteoclasts.

The enlarged surfaces of osteoid tissue on the trabeculae were covered with osteoblast cells in the same proportion as normal - with the increased osteoid tissue abundance, however, the osteoblasts were increased in number.

There was a pattern of sandwiched structures of osteoid tissue and mineralized bone - in some instances entirely unmineralized trabeculae. This change was pronounced to a degree not compatible with any other known bone condition.

With the quantitative electron microscopy technique the calcium content was measured in a series of samples with changes typical of post-traumatic osteopenia. It was discovered that even if the colour changes, in histochemical preparations designed to measure osteoid, were sharp, the actual mineralization changed gradually through the trabeculae.

With quantitative measurements of small areas located centrally in the trabeculae it was found that in trabeculae from patients with post-traumatic osteopenia the calcium content could vary from normal to a reduction by 100%

An additional study included the roentgen examination of the

femora of patients who had, in the past (2-17 years earlier) sustained a tibia shaft fracture. There was an approximately 10% thinning of the cortices, resulting in a slight widening of the marrow cavity which did not change in time.

Post-traumatic osteopenia is,from the morphological point of view, a high turn-over condition of several years' duration, which finally reaches a steady state, usually with a small net loss of bone.

REFERENCES

Andersson, S. (1978) Activity, inactivity and fracture. Thesis, University of Lund, Sweden.

Andersson, S. & Nilsson, B.E. (1979a) Changes in bone mineral content following tibial shaft fractures. Clin. Orthop.144:226-229.

Andersson, S. & Nilsson, B.E. (1979b) Post-traumatic bone mineral loss in tibial shaft fractures treated with a weight-bearing brace. Acta orthop. scand. 50:689-691.

Arnold, J.S. & Jee, W.S.S. (1954) Embedding and sectioning undecalcified bone and its application to radioautography. Stain Technology 29:225-239.

Bach Christensen, S. & Arnoldi, C.C. (1980) Distribution of ^{99m}Tc-phosphate compounds in osteoarthritic femoral heads. J. Bone Jt Surg. 62-A:90-96.

Baron, R., Vignery, A., Neff, L., Silverglate, A., Santa Maria, A. (1983) Processing of undecalcified bone specimens for bone histomorphometry. In: Bone Histomorphometry. Techniques and interpretation. Ed: Recker, R. Publ. CRC Press, Florida, pp 13-35.

Baud, C.-A. & Pouëzat, J.-A. (1973) Données microradiographiques quantitatives sur le minéral osseux dans l'ostéoporose posttraumatique. Rhumatologie 25:75-77.

Bauer, G.C.H. & Carlsson, A. (1955) Post-fracture bone salt resorption studied in rats. Acta orthop. scand.25:83-88.

Beck, O. (1925) Die pathologische Anatomie und spezielle Pathologie der Knochenatrophie. Ergeb. Chir. Orthop. 18:556.

Bélanger, L.F. (1969) Osteocytic osteolysis. Calc. Tiss. Res. 4:1-12.

Belenger, M. (1956) Aspect clinique et méthodes thérapeutiques. L'Ostéoporose posttraumatique. Dixième Congrès Belge de Chirurgie, Knokke.

Bohr, H. & Sørensen, A.H. (1950) Study of fracture healing by means of radio-active tracers. J. Bone Jt Surg. 32-A:567-574.

Bunch, T.E., Young, D.R., Niklowitz, W.Y. (1982) Disuse osteoporosis in the monkey: Electron probe analysis of cortical bone. Proc. Amer. Soc. Bone and Mineral Res., p.3.

Dalén, N. & Olsson, K. E. (1974) Bone mineral content and physical

activity. Acta orthop. scand. 45:170-174.

Danielsson, L.G., Dymling, J.F. & Heripret, G. (1964) Coxarthrosis in man studied with external counting of ^{85}Sr and ^{47}Ca. Clin. Orthop. 31:184-199.

Eichler, J.H. (1970) Inaktivitätsosteoporose. Georg Thieme Verlag, Stuttgart.

Exner, A. (1902) Beiträge zur Kenntnis der akuten Knochenatrophie. Fortschr. Röntgenstr. 6:1-12.

Frank, R., Kern, R. & Fontaine, R. (1957) Etude histopathologique au microscope électronique de l'ostéoporose post-traumatique. Path. et Biol. 5:2203-2208.

Freudiger, von A. (1950) Die pathologische mechanische Konstellation als ätiologischer Faktor der Sudeckschen Knochenatrophie nach Frakturen. Helv. chir. Acta 17:426-448.

Frost, H.M. (1959) Staining of fresh, undecalcified, thin bone sections. Stain Technol. 34:135-146.

Frost, H.M. (1969) Tetracycline-based histological analysis of bone remodeling. Calc. Tiss. Res. 3:211-237.

Geiser, M. & Trueta, J. (1958) Muscle action, bone rarefaction and bone formation. J. Bone Jt Surg. 40-B:282-311.

Grynpas, M.D. (1983) Changes in bone mineral structure in immobilized monkeys. Calc. Tiss. Int. 35:662.

Herfarth, H. (1924) Beitrag zur Frage der Sudeck'schen Knochenatrophie. Bruns' Beitr. klin. Chir.132:165-190.

Jaffe, H.L. (1930) The resorption of bone. Arch. Surg. 20:355-385.

Johnell, O. (1980) The clinical significance of osteoclast activity in man. Thesis, University of Lund, Sweden.

Johnell, O. (1984) Personal communication.

Johnell, O., Wiklund, P.E., Hulth, A. (1977) Osteoclast counting in crista biopsies. Acta orthop. scand. 48:566-571.

Kharmosh, O. & Saville, P.D. (1965) The effect of motor denervation on muscle and bone in the rabbit's hind limb. Acta orthop. scand. 36:361-370.

Kienböck, R. (1901) Ueber acute Knochenatrophie bei Entzündungsprocessen an den Extremitäten (fälschlich sogenannte Inactivitätsatrophie der Knochen) und ihre Diagnose nach dem Röntgen-Bilde. Wien. med. Wschr. 28:1345-1348.

Kilian, H.F. (1857) Das halisteretische Becken... Bonn.

Kühr, J. (1982) Der Einfluss der Immobilisation auf Zahl und Funktion der Osteoblasten. Chirurg 53:160-164.

Lacroix, P. & Ponlot,R. (1956) Remarques sur l'histopathologie de l'ostéoporose posttraumatique. Acta chir. belg. Acta orthop. belg. Suppl. 1:149-164.

Landoff, G. (1942) Experimental investigations on bone atrophy in connection with immobilization (plaster) and acute arthritis. Acta chir. scand. Suppl. 71.

Landry, M. & Fleisch, H. (1964) The influence of immobilization on bone formation as evaluated by osseous incorporation of tetracyclines. J. Bone Jt Surg. 46-B:764-771.

Mattsson, S. (1972) The reversibility of disuse osteoporosis. Acta orthop. scand. Suppl. 144.

Melsen, F., Melsen, B., Mosekilde, L. & Bergmann, S. (1978) Histomorphometric analysis of normal bone from the iliac crest. Acta path. microbiol. scand. Sect. A. 86:70-81.

Merz, W.A. & Schenk, R.K. (1970) A quantitative histological study on bone formation in human cancellous bone. Acta anat. 76:1-15.

Meyer, A. (1960) Recherches sur l'infrastructure du tissue osseux. Faculté de Médicine de Strasbourg. 29:11-56.

Minaire, P., Meunier, P., Edouard, C., Bernard, J., Courpron, P.& Bourret. J. (1974) Quantitative histological data on disuse osteoporosis. Calcif. Tiss. Res. 17:57-73.

Nilsson, B.E. (1966) Post-traumatic osteopenia. A quantitative study of the bone mineral mass in the femur following fracture of the tibia in man using Americium-241 as a photon source. Acta orthop. scand. Suppl. 91.

Nilsson, B.E. & Westlin, N.E. (1971) Bone density in athletes. Clin. Orthop. 77: 179-182.

Obrant, K. (1984) Tetracycline double-labelling in post-traumatic osteopenia in man. Arch. Orthop. Trauma Surg. In press.

Raina, V. (1973) Rickets and osteomalacia - A morphological study. Indian J. Med. Res. 61:190-194.

Rieder, W. (1936) Die akute Knochenatrofie. Dtsch. Z. Chir. 248: 269-331.

Ronse, E. (1963) L'osteoporose post-traumatique chez l'adulte.

Documents Radiographiques. Louvain, Imprimerie Sintal.

Rosenberg, G. & Simmons, D.J. (1979) Biological rhythms in dentin calcification. Calcif. Tiss. Int. 28:163.

Sissons, H.A. (1962) Age changes in the structure and mineralization of bone tissue in man. Radio-Isotopes and bone. Symposium Princeton. Ed: F.C.McLean, Oxford, pp 443-465.

Steinbach, H.L. (1964) The roentgen appearance of osteoporosis. Radiol. Clin. N. Amer. 2:191-207.

Sudeck, P. (1900) Ueber die acute entzündliche Knochenatrophie. Arch. klin. chir. 62:147-156.

Uhthoff, H.K. & Jaworski, Z.F.G. (1978) Bone loss in response to long-term immobilisation. J. Bone Jt Surg. 60-B:420-429.

Wendeberg, B. (1961) Mineral metabolism of fractures of the tibia in man studied with external counting of Sr^{85}. Acta orthop. scand. Suppl. 52.

Westlin, N. (1974) Bone mineral in Colles' fracture. Opuscula Medico-Technica Lundensia XII.

Wiklund, P.E. (1980) The clinical significance of unmineralized bone matrix in man. Thesis, University of Lund, Sweden.

Volkmann, R. (1862) Chirurgische Erfahrungen über Knochenverbiegungen und Knochenwachsthum. Virchows Arch. path. Anat. 24:512-540.

Wredmark, T. (1977) Reaction of the growing rat skeleton to immobilisation and fracture. Thesis, Karolinska Institute, Stockholm, Sweden.

PAPERS

Acta Orthop Scand 55, 78–82, 1984

Trabecular bone changes in the greater trochanter after fracture of the femoral neck

Bone samples were taken from the trabecular part of the greater trochanter in 32 patients who had had a fracture of the ipsilateral femoral neck, and from 24 patients who had coxarthrosis. 42 cadavers served as controls. The samples were sectioned, stained and examined histologically.

The coxarthrosis cases differed only slightly from normal, whereas the fracture cases had increased osteoid volume and surface. Osteoclasts were also increased in number, as were active osteoblasts.

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Key words: bone; femur; fracture; osteoarthritis; osteoporosis.

Herfarth (1924) and Beck (1925) found increasing numbers of osteoclasts in bone biopsies following trauma. Rieder (1936) also described an increasing fraction of unmineralized bone after fracture in man. Freudiger (1950) and Geiser & Trueta (1958) found the same in animal experiments. Landoff (1942), however, in rabbits found no increase in the amount of unmineralized bone. None of these early investigators had quantified the histological changes in bone. In addition, all the examinations were undertaken before the technique of cutting and staining undecalcified sections became available.

The histomorphometric analysis method described by Merz & Schenk (1970) has made it possible to quantify histological entities in an undecalcified bone section. Although this histological technique has been available for decades, most investigations on post-traumatic osteopenia have been non-invasive. In immobilized rats, Eichler (1970) found increasing osteoid volume, increasing numbers of osteoclasts and decreasing numbers of osteoblasts. On the other hand, Minaire et al. (1974) in iliac crest biopsies from immobilized patients found osteoporosis and no increasing amount of osteoid. Baud & Pouëzat (1973) described two cases of post-traumatic osteopenia in which, using the quantitative micro-radiographic technique, they found a lowered mineral content in the trabeculi. Dempster et al. (1980) have shown by the electron-probe technique that the concentration of calcium differs between parts of the trabeculi. There was a difference between not only osteoid and mineralized bone, but also within the osteoid and within the mineralized bone. In addition, the low correlation between the absolute volume of trabecular bone and ash-weight in iliac bone samples (Melsen & Mosekilde 1981) indicates variations in the degrees of mineralization of trabecular bone also in man.

The aim of the present investigation was to examine morphologic changes in the trabecular bone after fracture.

Patients and methods

Fracture cases

The 32 patients selected for the investigation had all fractured the femoral neck 8±10 months earlier. Their age at the time of the biopsy was 75 (52–93) years (Table 1) and they were all scheduled for reconstruction surgery.

Table 1. Age and sex

	Men		Women	
	N	Age	N	Age
Femoral neck fracture	10	75±12	22	75± 8
D:o controls (AVTB%)	11	75± 7	12	75±11
Coxarthrosis	9	71± 5	15	70± 7
D:o controls (AVTB%)	14	71±10	13	70±12
Controls, all	25	53±23	17	63±23

AVTB%: Absolute volume of trabecular bone.

Coxarthrosis

Twenty-four consecutive patients with primary coxarthrosis were selected. Their age at the time of the biopsy was 71 (56–79) years, and they were all to have total hip replacement procedures.

Controls

Bone samples were taken from 42 necropsy cases in the Department of Forensic Medicine, all from individuals who had suffered sudden death. In each case, samples were obtained from both greater trochanters. The age was 57 (16–95) years. Cases with known alcoholism or malignant disease were not included in either group.

Biopsy

A biopsy was taken from the trabecular bone in the greater trochanter (Figure 1). In the control cases the biopsy technique and the instrument described by Burkhardt (1966) were used, resulting in a bone cylinder 15 mm long and 5 mm in diameter. In the coxarthrosis and fracture cases the biopsy was obtained from the same site with a chisel in conjunction with a hip arthroplasty.

Histological methods

The bone samples were fixed in 10% buffered formalin for at least 24 h and then dehydrated with alcohol in increasing concentrations (40, 70, 96, 99.9%), 24 h for each concentration. Defatting was carried out in a 3:1 solution of 99.9% alcohol and chloroform for another 24 h, followed by rinsing in xylol. The bone samples were then ready for embedding in methylmethacrylate, adding benzylperoxide and plastoid N in 37° for 48 h. Four of the 32 post-fracture cases were immediately fixed in 99% ethanol instead of formalin. These were samples from patients who had been given tetracycline preoperatively for a parallel study. The samples were then sectioned in a hard sectioning Jung microtome, model K, making sure that the sections for morphometric evaluation were collected from the central part of the specimen. Sections, 5 μm thick, were prepared and stained according to Goldner's modification of Masson trichrome staining (Goldner 1938).

Morphometric evaluation

For the morphometric evaluation, a template consisting of a point-wave pattern in the eye-piece of the microscope (Merz & Schenk 1970) was used. For the calculation of osteoid parameters, an objective magnification of 13× and an eye-piece magnification of 10× were used. With this magnification, 30 fields including only trabecular bone could be measured in most sections (Johnell et al. 1979). For the purpose of counting the osteoclasts, the margins of the sections were taped in order to obtain a straight-line rectangular area of trabecular bone. All osteoclasts in the area were counted per mm^2 with 40× objective and 10× eye-piece magnification. The osteoclasts were identified by the light pink, often vacuolar cytoplasm and the distinct nucleoli. Only osteoclasts with two or more nuclei and in contact with the bone surface were counted (Johnell et al. 1977). Active osteoblasts were defined as cuboidal cells in contact with osteoid, often with their nuclei in the part of the cytoplasm facing away from the trabeculum. For the counting of active osteoblasts an objective magnification of 40× and an eye-piece magnification of 12.5× were used, again with a point-wave template. Counting sites were selected at random. Intersections lined with active osteoblasts and intersections with other lining cells were counted.

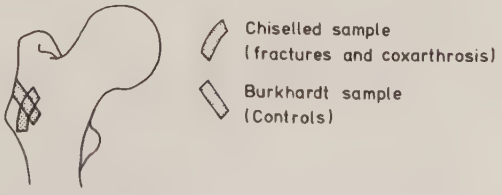

Figure 1. Sample sites.

The following variables were calculated:

1. Absolute volume of trabecular bone (AVTB%): the percentage of bone trabeculae, including mineralized as well as osteoid tissue, of the total bone volume, including medullary and vascular space.
2. Relative osteoid surface (OS%): the bone surface covered by osteoid expressed as a percentage of the total trabecular bone surface.
3. Relative osteoid volume (OV%): the percentage of AVTB% occupied by osteoid.
4. Osteoclast count: the number of osteoclasts/mm^2 section surface covered by trabecular bone.
5. Osteoblast ratio: the ratio of osteoid surface covered by active osteoblasts/osteoid lined by other cells.

For comparison of AVTB% only, age- and sex-matched controls were used. Since, for other variables no age or sex differences were suspected or could be demonstrated, all available controls were used.

Results

Since in the control cases there was no left-right difference in any of the variables, the data from both sides were pooled.

The absolute volume of trabecular bone was significantly lower in men with coxarthrosis than in controls; otherwise, there were no differences between the groups. No correlations with age were found. If all controls were included and compared between men and women, the AVTB% was significantly less in women ($p < 0.01$) (Table 2).

The osteoid surface in the fracture cases was increased by a factor of 14, but also in the coxarthrosis patients there was a significant increase ($p < 0.05$). Similarly, the osteoid volume was increased in the fracture cases ($p < 0.001$) (Table 3).

Table 2. Absolute volume of trabecular bone, per cent

	Men	Women
Femoral neck fracture	13.8±5.9 n.s.	17.6±10.8 n.s.
D:o controls	17.8±4.8	12.6± 4.7
Coxarthrosis	11.1±4.4 p<0.01	14.6± 8.7 n.s.
D:o controls	17.4±4.3	13.0± 4.9

The number of osteoclasts was significantly higher ($p < 0.01$) in the post-fracture cases compared with the control cases (Table 3).

Finally, active osteoblasts also occurred more often in fracture patients than in controls ($p < 0.01$).

A frequent finding was that osteoid and bone (n.b. layers more and less mineralized) could be observed; this was altogether different from the appearance in, for instance, osteomalacia (Figure 2).

Discussion

The absolute volume of trabecular bone varied in an inconsistent manner. This variable is usually difficult to handle because of its large variation, but nevertheless – in the separate sample – it is a necessary correction variable. The most obvious finding in this study was the increased amount of osteoid tissue in the post-traumatic cases. In many trabeculi there was an arrangement with unmineralized bone lamellae alternating with mineralized bone lamellae. Raina (1973) described mineralized bone on the surface of osteoid in renal osteomalacia, but this phenomenon, although probably fairly common, has not otherwise been described previously. Methodological explanations may be found in the staining pro-

Table 3. Osteoid, osteoclasts and osteoblasts

	Relative osteoid surface %	Relative osteoid volume %	Osteoclast count/mm^2	Osteoblast ratio
Controls	1.2± 1.4	0.1±0.3	0.16±0.22	0.39±0.35
Coxarthrosis	2.9± 3.9	0.5±1.1	0.22±0.27	0.51±0.41
Femoral neck fracture	17.3±16.2	3.5±3.8	0.48±0.73	0.65±0.31

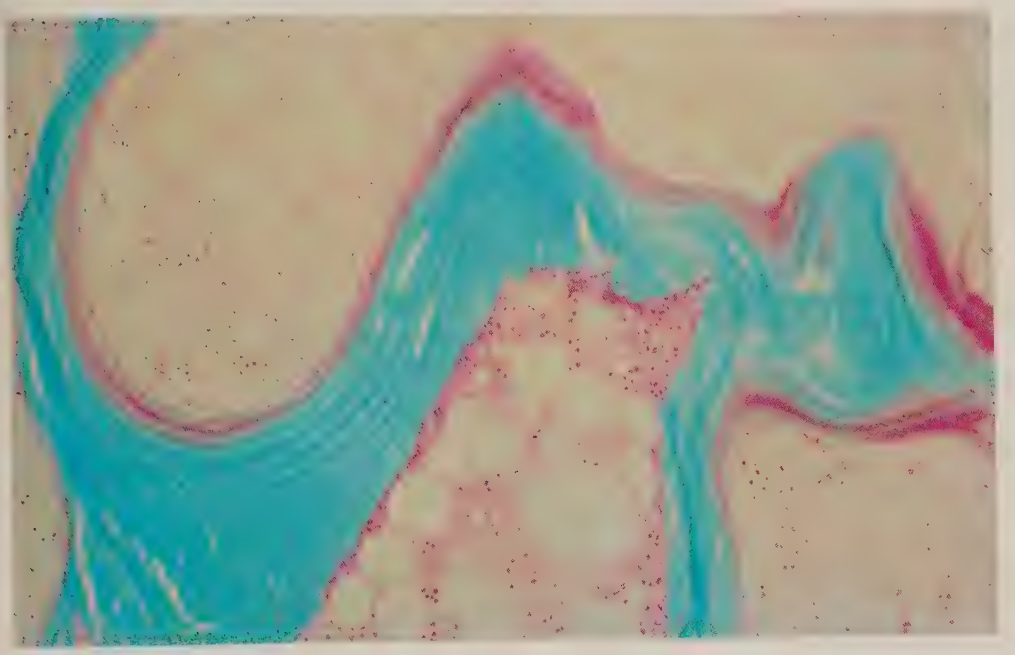

Figure 2. Goldner-stained sample from patient with femoral neck fracture (110×). Layers of osteoid (red) and mineralized bone (green). Also, the mineralized part of the trabeculum has a lamellar appearance – this type of lamellae may be seen in normal bone.

cedures, the microscopic techniques (Olah 1980) and the section thickness. However, these mineralization defects in the post-traumatic cases could not be a staining artefact as they were not seen in any of the control samples which were handled together with the fracture patient samples.

What is the significance of the increased osteoid in the fracture cases? In the past we have failed to demonstrate serious osteomalacia in hip fracture patients, at least to this extent (Hodkinsson 1971, Lips 1982). Since there are no other data on osteoid from this location, we must accept the findings at their face value.

The increase in osteoid and the unusual pattern of mineralization are probably signs of high turnover disturbance of the local bone metabolism rather than an osteomalacia lesion. Again, the presence of unmineralized bone may explain the capacity to recover lost bone mineral (Nilsson 1966, Westlin 1974, Andersson & Nilsson 1979).

The number of osteoclasts was tripled in the post-traumatic cases compared with the controls. This is most likely a sign that bone resorption is taking place, just as the increased osteoblast count indicates increased bone formation.

The coxarthrosis samples also deviated from the controls. In coxarthrosis there is an ongoing repair process in the upper end of the femur, as demonstrated by Danielsson et al. (1964) and others.

In conclusion, the data from the present study support the earlier findings of an increased metabolic rate in a fractured limb (Wendeberg 1961) in man; the cellular activities suggest that resorption and formation are both increased. In addition, there is an arrangement of more and less mineralized lamellae, which is unusual in other conditions.

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References

Andersson, S. M. & Nilsson, B. (1979) Changes in bone mineral content following tibia shaft fractures. *Clin. Orthop.* **144**, 226–229.

Baud, C. A. & Pouëzat, J. A. (1973) Données microradiografiques quantitatives sur le minéral osseux dans l'osteoporose traumatique. *Rhumatologie (Aix-les-Bains)* **25**, 75–77.

Beck, O. (1925) Die pathologische Anatomie und spezielle Pathologie der Knochenatrophie. *Ergeb. Chir. Orthop.* **18**, 556–689.

Burkhardt, R. (1966) Technische Verbesserungen und Anwendungsbereich der Histo-Biopsie von Knochenmark und Knochen. *Klin. Wochenschr.* **44**, 326–334.

Danielsson, L. G., Dymling, J. F. & Heripret, G. (1964) Coxarthrosis in man studied with external counting of ^{85}Sr and ^{47}Ca. *Clin. Orthop.* **31**, 184–199.

Dempster, D. W., Elder, H. Y., Nicholson, W. A. P., Smith, D. A. & Moss, V. A. (1980) Changes in bone mineral in experimentally induced rickets in the rat: electron microprobe and chemical studies. *Calcif. Tiss. Int.* **30**, 135–146.

Eichler, J. H. (1970) *Inaktivitätsosteoporose.* Georg Thieme Verlag, Stuttgart.

Freudiger, A. (1950) Die pathologische mekanische Konstellation als ätiologischer Faktor der Sudeckschen Knochenatrophie nach Frakturen. *Helv. Chir. Acta* **17**, 426–448.

Geiser, M. & Trueta, J. (1958) Muscle action, bone rarefaction and bone formation. *J. Bone Joint Surg.* **40**-B, 282–311.

Goldner, J. (1938) A modification of the Masson trichrome technique for routine laboratory purpose. *Am. J. Pathol.* **14**, 237–243.

Herfarth, H. (1924) Beitrag zur Frage der Sudeckschen Knochenatrophie. *Brun. Beitr. Klin. Chir.* **132**, 165–190.

Hodkinson, H. M. (1971) Fracture of the femoral neck in the elderly. Assessment of the role of osteomalacia. *Gerontol. Clin.* **13**, 153–158.

Johnell, O., Wiklund, P. E. & Hulth, A. (1977) Osteoclast counting in crista biopsies. *Acta Orthop. Scand.* **48**, 566–571.

Johnell, O., Nilsson, B. E., Wallöe, A. & Wiklund, P. E. (1979) Bone morphology in epileptics. *Calcif. Tiss. Int.* **28**, 93–97.

Landoff, G.-A. (1942) Experimentelle Untersuchungen über die Knochenatrophie infolge einer Immobilisation und einer akuten Arthritis. *Acta Chir. Scand.* Suppl. 71.

Lips, P. (1982) *Metabolic causes and prevention of femoral neck fractures.* Thesis, Amsterdam.

Melsen, F. & Mosekilde, L. (1981) The role of bone biopsy in the diagnosis of metabolic bone disease. *Orthop. Clin. N. Am.* **12**, 571–602.

Merz, W. A. & Schenk, R. K. (1970) Quantitative structural analysis of human cancellous bone. *Acta Anat. (Basel)* **75**, 54–66.

Minaire, P., Meunier, P., Edouard, C., Bernard, J., Courpron, P. & Bourret, J. (1974) Quantitative histological data on disuse osteoporosis. *Calcif. Tiss. Res.* **17**, 57–73.

Nilsson, B. (1966) Post-traumatic osteopenia. *Acta Orthop. Scand.* Suppl. 91.

Olah, A. J. (1980) Effects of microscopic resolution on histomorphometrical estimates of structural and remodelling parameters in cancellous bone. *Pathol. Res. Pract.* **166**, 313–322.

Raina, V. (1973) Rickets and osteomalacia – A morphological study. *Indian J. Med. Res.* **61**, 190–194.

Rieder, W. (1936) Die akute Knochenatrophie. *Dtsch. Z. Chir.* **248**, 269–331.

Wendeberg, B. (1961) Mineral metabolism of fractures of the tibia in man studied with external counting of Sr^{85}. *Acta Orthop. Scand.* Suppl. 52.

Westlin, N. E. (1974) Loss of bone mineral after Colles' fracture. *Clin. Orthop.* **102**, 194–199.

II

HISTOMORPHOLOGIC CHANGES IN THE TIBIAL EPIPHYSIS AFTER DIAPHYSEAL FRACTURE

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ABSTRACT

The amount of osteoid tissue, osteoclasts, and active osteoblasts was measured in biopsy specimens from the proximal end of the tibia in 20 patients who had sustained tibial shaft fractures. As compared with samples from 42 control subjects without fractures, there was a vast increase in the amount of osteoid tissue. The histologic appearance deviated from that of other skeletal conditions in that poorly calcified (osteoid) layers were found throughout the trabeculae, which, in some instances, were completely osteoid. The frequency of active osteoblasts (corrected for osteoid) was not increased in the patients with fractures, whereas osteoclasts were noted four times more frequently.

INTRODUCTION

In 1902 Exner[9] examined post-traumatic bones histologically and chemically. He found decreasing numbers of trabeculae and low ash weights. Other early investigators of post-traumatic osteopenia were Herfarth[16] and Beck.[3] They found increased numbers of osteoclasts.Rieder[24] found an increased fraction of unmineralized bone after fracture in humans. Corresponding animal experiments gave the same results.[11,14] Landoff,[19] however,found no increase in the amount of unmineralized bone in rabbits. None of these early investigators quantified the histologic changes in bone following trauma. In addition, the examinations were undertaken before the technique for cutting and staining undecalcified sections became available.

The histomorphometric analysis method described by Frost[12] and Merz and Schenk[21] has made it possible to quantify histologic entities in an undecalcified bone section. Eichler[8] found increased osteoid volume, an increased number of osteoclasts, and a decrease in osteoblasts in immobilized rats. Baud and Pouezat[2] described two patients with post-traumatic osteopenia in whom they found lowered mineral content in the trabeculae by a quantitative microradiographic technique. Dempster et al.,[7] by an electron probe technique, demonstrated varying concentrations of calcium in different parts of the trabeculae in animals. These differences were observed not only between osteoid and mineralized bone but also within the osteoid and the mineralized bone. The poor correlation between ash content and volume of trabecular bone[20] indicates variation in the degree of mineralization of trabecular bone in humans as well.

The aim of the present investigation was to perform histomorphometric and statistical evaluation of the changes occurring in the trabecular part of a long bone in humans after fracture of the diaphysis of the same bone.

MATERIAL AND METHODS

Patients

The patients selected for investigation had fractured the tibial diaphysis 0.5 - 152.0 months before the biopsy was performed (Figures 1 and 2). The age at the time of the biopsy was 34 $\pm$ 15 years (range 16 - 61 years). The distance between the biopsy site and the most proximal roentgenographically visible part of the fracture was 19 $\pm$ 6 cm. Nineteen patients had been scheduled for secondary surgical treatment of the tibia shaft fracture, which included the introduction of an intramedullary nail from the region of the tibial tuberosity; in one patient a Rush pin was extracted from the same region. In 12 of the patients a nail for external or internal fixation had been inserted in the upper end of the tibia.

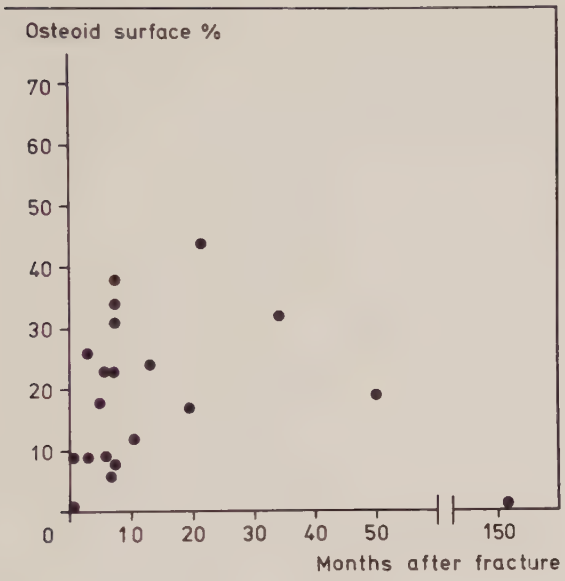

Figure 1. Osteoid surface in relation to time after fracture.

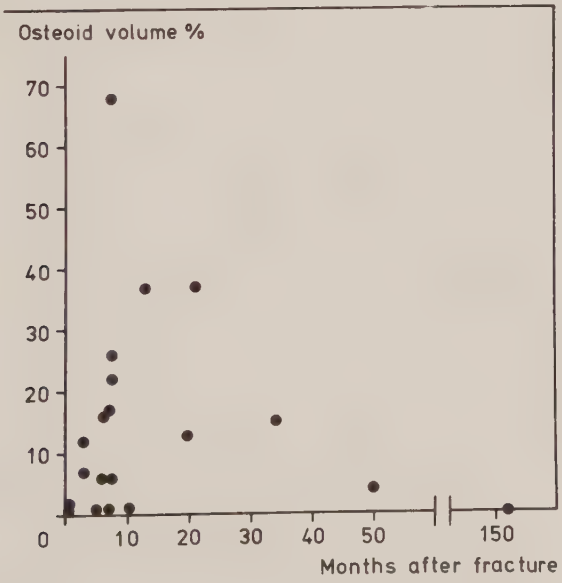

Figure 2. Osteoid volume in relation to time after fracture.

Control subjects

Samples were obtained bilaterally from the same site of the proximal end of the tibia from 42 subjects who had died suddenly and undergone autopsy. Subjects with known alcoholism or malignancy were excluded.

Biopsy

Bone biopsy specimens were obtained from the proximal tibial epiphysis (Figure 3). The biopsy technique and the instrument described by Burkhardt[6] were used, resulting in a bone cylinder 0.5 cm in diameter and approximately 1.5 cm long.

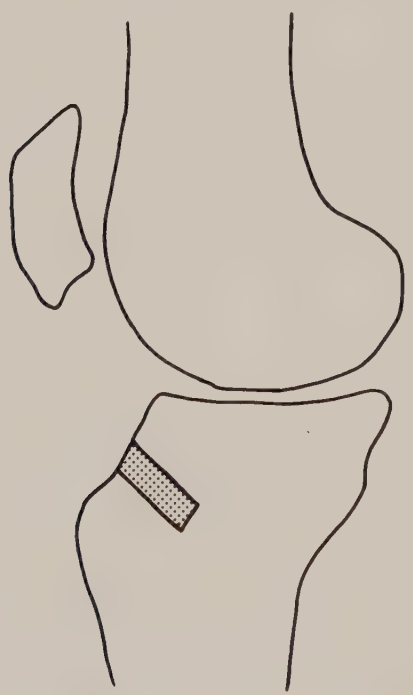

Figure 3. Biopsy site.

Histologic methods

The bone samples were fixed in 10% buffered formalin for at least 24 hours and then dehydrated with alcohol in increasing concentrations (40%, 70%, 96%, 99.9%) for an additional 24 hours for each concentration. Defatting was performed in a 3/1 solution of 99.9% alcohol and chloroform for 24 hours, followed by rinsing in xylol. The bone samples were then ready for embedding in methylmethacrylate with the addition of benzylperoxide and plastoid N* at 37° for 48 hours.

The samples were sectioned in a hard-sectioning Jung microtome (model K); care was taken to ensure that the sections for morphometric evaluation were collected from the central part of the specimen. For the evaluation of bone volume, osteoid parameters, osteoclast count, and osteoblast ratio, 5 µm sections were prepared and stained according to Goldner's[15] modification of the Masson trichrome staining method.

Histomorphometric evaluation

For histomorphometric evaluation a template consisting of a point-wave pattern[21] was used, and for the calculation of osteoid parameters an objective magnification of x13 and an eye-piece magnification of x10 were used. With this magnification 30 fields that included only trabecular bone could be measured in most sections.[18] For the purpose of counting the osteoclasts, the margins of the sections were taped to obtain a rectangular area of trabecular bone. All osteoclasts in this area were counted (per mm^2) with x40 objective and x10 eye-piece magnifications. The osteoclasts were identified by the light pink, often vacuolar cytoplasm and the distinct nucleoli. Only osteoclasts with two or more nuclei that were in contact with the bone surface were counted.[17] Active osteoblasts were defined as cuboidal cells in contact with osteoid, often with the nuclei in the part of the cytoplasm facing away from the trabecula. For the counting of cuboidal osteoblasts an objective magnification of x40 and an eye-piece magnification of x12.5 were used, again with the Merz grid. Counting sites were selected at random. Intersections lined

* Röhm Pharma

with cuboidal osteoblasts and intersections with other lining cells were counted.

The following variables were calculated:

1. Absolute volume of trabecular bone (AVTB%), i.e. the percentage of bone, including mineralized and osteoid tissue,of the total bone volume, including medullary and vascular space.
2. Relative osteoid surface (OS%), i.e. the bone surface covered by osteoid, expressed as a percentage of the total trabecular bone surface.
3. Relative osteoid volume (OV%), i.e. the percentage of AVTB% occupied by osteoid.
4. Osteoclast count, i.e. the numbers of osteoclasts per mm^2 covered by trabecular bone.
5. Osteoblast ratio, i.e. the ratio of osteoid surface covered by cuboidal osteoblasts to osteoid lined by other cells.

For comparisons of AVTB% only age- and sex-matched control subjects were used. Since for other variables no influence of age or sex was suspected or demonstrated, all available control subjects were used.

RESULTS

Since there was no left-right difference in any of the variables, the data from both control tibiae were pooled. The trabecular bone volume in the proximal end of the tibia was reduced by approximately 20% in the patients with fractures, but the difference was not significant (Table 1). The percentage of the trabecular surface that was covered by osteoid was increased by a factor of 24 in the patients with fractures as compared with the control subjects (Table 2). The percentage of trabecular bone volume that consisted of unmineralized bone matrix (osteoid) was more than 100 times greater in the patients with fractures than in the control subjects.In relation to the time elapsed after fracture, both parameters of osteoid appear to be highest a few months to a few years after injury, but the time variable was too skewed to allow further analysis (Figures 1 and 2). Patients in

whom the upper end of the tibia had been tampered with did not deviate from the others in osteoid abundance.

Table 1. Absolute volume of trabecular bone (%)

	Men			Women		
	No	AVTB%	Age(yrs)	No	AVTB%	Age(yrs)
Patients with fractures	16	11.3 ± 5.6	29 ± 12	4	10.1 ± 7.8	53 ± 7
Control subjects (age-matched)	10	13.9 ± 3.3	28 ± 8	12	13.5 ± 4.6	54 ± 20
Control subjects (all)	25	14.7 ± 5.0	53 ± 23	17	12.5 ± 4.3	63 ± 23

Table 2. Osteoid and cellular components

	Relative osteoid Surface (%)	Relative osteoid Volume (%)	Osteoclast count/ mm^2	Osteoblast ratio
Patients with fractures	19.2 ± 12.5 ($p < 0.001$)	14.5 ± 17.1 ($p < 0.001$)	0.38 ± 0.63 ($p < 0.01$)	0.52 ± 0.25 NS
Control subjects	0.8 ± 1.1	0.12 ± 0.29	0.09 ± 0.15	0.53 ± 0.38

Osteoclasts were approximately four times as common in the samples from patients with fractures, whereas for any given amount of osteoid, cuboidal osteoblasts were equally common in fracture and control samples. A striking finding was the lamellar arrangement of the more calcified and the less calcified osteoid bone tissue (Figure 4). Uncalcified or slightly calcified lamellae were found at any depth in a trabecula, and in some instances entire trabeculae were lamellar or completely osteoid in their staining. In addition, spotted osteoid-type staining was observed.

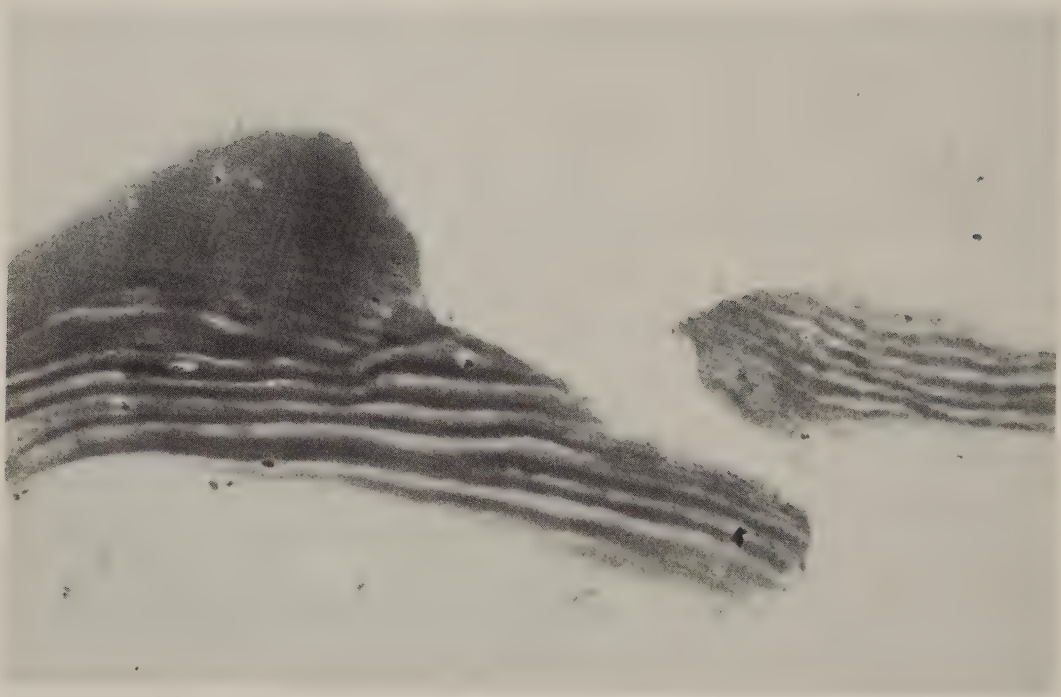

Figure 4. Lamellar structure of trabecula. The darker areas, which represent osteoid, appear to be sandwiched throughout most of the trabecula.

DISCUSSION

By gamma-absorptiometry Andersson and Nilsson[1] found a reduction of 40% or more in bone mineral content in the proximal end of the tibia after tibial shaft fractures. In the present study the reduction was considerably less. There is, however, no contradiction between the two studies: an additional 15% should be deducted from the data of the present study, since approximately that fraction was unmineralized or had a low degree of mineralization and appeared as osteoid in the samples. The gamma-absorptiometer does not record bone tissue without mineral, which may explain the discrepancy. Again, bone volume measurements are subject to considerable variation and difficult to handle in small samples.

The increased osteoclast count must be regarded as a sign of bone

resorption. However, osteoclasts were not found in all samples: only seven of the 20 patients with fractures had a positive finding of osteoclasts. In all but one of these patients a biopsy had been performed within the first year. There was no significant increase in cuboidal osteoblasts in the patients with fractures as compared with the control group. However, osteoblasts were rare since osteoid, particularly in the control group, was rare. The total number of osteoblasts, uncorrected for osteoid, was vastly increased in samples from patients with fractures. In conclusion, post-traumatic osteopenia has the histomorphology of a high turn-over lesion.

The most spectacular finding in samples from patients with tibial shaft fractures was, however, the irregularity of the osteoid, i.e., some trabeculae remained intact, some were partially osteoid, and some were completely uncalcified, all in a random pattern. Since these samples were handled with those of the control subjects, there is no reason to suspect bias with regard to staining or handling of the samples. The findings of this study are similar to those of Obrant[23] concerning trabecular structures near femoral neck fractures but much more pronounced. It appears that the bone trabeculae that are reduced only slightly in total volume have not become calcified or have by some mechanism of direct exchange become decalcified in the process. Frank et al.[10] Meyer,[22] Bohatirchuk,[4,5] and others have postulated that acellular calcium resorption from bone, i.e., "calciolysis," is a reality. This could be an alternative explanation if some generations of bone are more susceptible to calciolysis than others. Again, with a high turn-over bone metabolism bone tissue that has not been sufficiently calcified for detection by this staining technique may be expected. These findings are in agreement with the "regional accelerating phenomenon" proposed by Frost.[13] In addition, radioisotope studies indicate that post-traumatic osteopenia appears to be a high turn-over condition.[25] Unlike generalized osteopenia, i.e., postmenopausal or senile, which may be reversible only to a small extent, there is evidence of a considerable ability for restoration of bone mineral lost after fracture.[1] The presence of large amounts of unminera-

lized or poorly mineralized bone tissue explains this reversibility.

This micromorphologic appearance has not been encountered in studies of other metabolic bone diseases, including severe renal osteomalacia. However, the findings also introduce some doubt concerning previous interpretations of the histomorphometric technique, including the interpretation of Goldner[15] staining.

REFERENCES

1. Andersson, S.M. and Nilsson, B.: Changes in bone mineral content following tibia shaft fractures. Clin. Orthop. 144:226. 1979.
2. Baud, C.A. and Pouëzat, J.A.: Données microradiographiques quantitatives sur le minéral osseux dans l'ostéoporose traumatique. Rhumatologie 25:75, 1973.
3. Beck, O.: Die pathologische Anatomie und spezielle Pathologie der Knochenatrophie. Ergeb. Chir. Orthop. 18:556, 1925.
4. Bohatirchuk, F.: Calciolysis as the initial stage of bone resorption: A stain historadiographic study. Am. J. Med. 41:836, 1966.
5. Bohatirchuk, F.: Studies in bone formation and osteoid. Proc. 1st Workshop on Bone Morphometry, University of Ottawa, March 28-31,1973, p. 197.
6. Burkhardt, R.: Technische Verbesserungen und Anwendungsbereich der Histo-Biopsie von Knochenmark und Knochen. Klin. Wochenschr. 44:326, 1966.
7. Dempster, D.W., Elder, H.Y., Nicholson, W.A.P., Smith, D.A. and Moss, V.A.: Changes in bone mineral in experimentally induced rickets in the rat: Electron microprobe and chemical studies. Calcif.Tissue Int. 30:135, 1980.
8. Eichler, J.H.: Inaktivitätsosteoporose. Stuttgart, Georg Thieme Verlag, 1970, pp. 1-87.
9. Exner, A.: Beiträge zur Kenntnis der akuten Knochenatrofi.Fortschr. Röntgenstr. 6:1, 1902.
10. Frank, R.M., Kern, R. and Fontaine, R.:Etude histopathologique au microscope électronique de l'ostéoporose posttraumatique.

Pathol.Biol. 5:2203, 1957.

11.Freudiger, A.: Die pathologische-mekanische Konstellation als ätiologischer Faktor der Sudeckschen Knochenatrophie nach Frakturen. Helv. Chir. Acta 17:426, 1950.

12.Frost, H.M.: Tetracycline-based histological analysis of bone remodelling. Calcif. Tissue Res. 3:211, 1969.

13.Frost, H.M.: A chondral modelling theory. Calcif. Tissue Int. 28:181, 1979.

14.Geiser, M. and Trueta, J.: Muscle action, bone rarefaction and bone formation. J. Bone Jt Surg. 40 B:282, 1958.

15.Goldner, J.: A modification of the Masson trichrome technique for routine laboratory purpose. Am. J. Pathol. 14:237, 1938.

16.Herfarth, H.: Beitrag zur Frage der Sudeckschen Knochenatrophie. Bruns Beitr. Klin. Chir. 132:165, 1924.

17.Johnell, O.,Nilsson, B.E., Wallöe, A. and Wiklund, P.E.: Bone morphology in epileptics. Calcif. Tissue Int. 28:93, 1979.

18.Johnell, O.,Wiklund, P.E. and Hulth, A.: Osteoclast counting in crista biopsies. Acta Orthop. Scand. 48:566, 1977.

19.Landoff, G.-A.: Experimentelle Untersuchungen über die Knochenatrophie infolge einer Immobilisation und einer akuten Arthritis.Acta Chir. Scand. Suppl. 71, 1942.

20.Melsen, F. and Mosekilde, L.: The role of bone biopsy in the diagnosis of metabolic bone disease. Orthop. Clin. North Am. 12:571, 1981.

21.Merz, W.A. and Schenk, R.K.: Quantitative structural analysis of human cancellous bone. Acta Anat. 75:54, 1970.

22.Meyer, A.: Récherches sur l'infrastructure du tissu osseux. Fac. Med. Strasbourg 29:11, 1960.

23.Obrant, K.J.: Trabecular bone changes in the greater trochanter after fracture of the femoral neck. Acta Orthop. Scand. 55:78,1984.

24.Rieder, W.: Die akute Knochenatrophie. Dtsch. Z. Chir. 248:269, 1936.

25.Wendeberg, B.: Mineral metabolism of fractures of the tibia in man studied with external counting of Sr^{85}. Acta Orthop. Scand. Suppl. 52, 1961.

III

ELECTRON MICROPROBE ANALYSIS AND HISTOCHEMICAL EXAMINATION OF THE CALCIUM DISTRIBUTION IN BONE TRABECULAE IN MAN. A METHODOLOGICAL STUDY, USING BIOPSY SPECIMENS FROM POST-TRAUMATIC OSTEOPENIA

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ABSTRACT

Energy dispersive X-ray microanalysis (EDX or electron microprobe analysis) of the relative concentration of calcium in different bone trabeculae from the tibia epiphysis, and in different parts of one and the same trabecula, was performed in three patients who had earlier had a fracture of the ipsilateral tibia diaphysis. The variation in calcium concentration was compared with the histochemical patterns obtained with both the Goldner and the von Kossa staining techniques for detecting calcium in tissue.

Previously reported calcium distribution features, found to be typical for post-traumatic osteopenia, such as sandwiched mineralization patterns in individual trabeculae and large differences in the mineralization level between different trabeculae, could be verified both by means of the two histochemical procedures and from the electron microprobe analysis. A pronounced difference, however, was observed between the two histochemical staining techniques as regards their sensitivity to detect calcium. The two methods have different turn-over points, from negative to positive, along a gradient change of calcium concentrations. To judge from the values obtained from the EDX-measurements, the sensitivity of the Goldner technique can be said to be about ten times higher than that of von Kossa.

The EDX measurements gave more detailed information than either of

the two histochemical techniques: great variations in calcium concentration were found both in trabeculae stained as unmineralized as well as mineralized.

INTRODUCTION

Loss of bone mineral after fracture in man has in the past been demonstrated mainly by radiometric techniques[1,2,3]. Such post-traumatic osteopenia in man has been studied with histological techniques on decalcified samples[4,5], as well as undecalcified tissue[6,7,8].A not unusual finding in these earlier investigations was an increased amount of osteoid. Baud and Pouëzat[8] suggested that after fracture the bone mineral content of entire trabeculae may be decreased. Recent studies have shown by means of histochemical techniques, that there is a large variation in the proportion of osteoid in bone after fracture. With the Goldner staining technique[9] poorly calcified areas, distributed in patches or sandwiched layers, may be found deep in the trabeculae[10,11]. With the von Kossa staining technique[12] there appears to be even less calcium in the bone trabeculae (Obrant, unpublished observations). As these mineralization characteristics are unique, and have only recently been described, the following questions are raised:

1. Are these mineralization characteristics real, or could they in some way be staining artifacts?
2. Are the incoherent results from different standard staining techniques due to a sensitivity-difference between these techniques?

The aim of this methodological study was thus to answer these questions by means of electron microprobe analysis of the calcium distribution in various parts of trabecular bone from patients with post-traumatic osteopenia, and further, to correlate the results of the measurements with observations made in histological sections stained with the Goldner and von Kossa procedures.

MATERIAL AND METHODS

Three men, aged 19 - 37, who had had a fracture of the tibia diaphysis, were chosen for the investigation. They were operated on because of delayed union or pseudarthrosis 7 - 13 months after the occurrence of the fracture. The fractures were stabilized by a marrow nail, making the proximal tibia epiphysis available for biopsy (Figure 1).The distance between the biopsy site and the most proximal part of the fracture was in no case less than 18 cm. The primary fracture treatment was in all three patients reduction and immobilization in a plaster cast. In one patient the fracture had also been stabilized with screws. The upper end of the tibia had not been interfered with. Bone cylinders with a diameter of 5 mm and an approximate length of 15 mm were obtained by means of a Burkhardt drill[13]. The samples were fixed in absolute ethanol for at least 24 hours, dehydrated in a 3/1 solution of absolute ethanol and chloroform for 24 hours, and rinsed in xylene. Finally, they were embedded in methylmethacrylate containing benzylperoxide and plastoid N at 37° C for 48 hours.

From each sample, three consecutive, 5 µm thick sections were cut longitudinally with a Jung hard-sectioning microtome. The central section was left unstained, mounted on an aluminum plate and carbon coated for analysis in a scanning-transmission (SEM-STEM) equipped electron microscope, Jeol 200 CX, connected to a Link 860 energy dispersive X-ray microanalyser. The other two sections were mounted on glass slides, one stained according to Goldner, and the other according to von Kossa, and examined in a light microscope. By this approach, single trabeculae could be identified in the electron microscope, and related to the two sections stained for light microscopical histochemical analysis.

Trabeculae that showed markedly different staining characteristics were chosen for further examination in the electron microscope. In two of the patients the measurements were performed in different trabeculae. In the third patient different parts of the same trabecula were analyzed. Of special interest were areas with osteoid, arranged in a sandwiched pattern, which were analysed

Figure 1. Schematic view showing the site of the fracture in all the three cases investigated, as well as the area where the biopsy specimen was taken.

stepwise along a line perpendicular to the striation in the corresponding stained section. The areas of the measured fields were set to c 0.05 μm^2, or 0.0125 μm^2, depending on the selected magnification. From the spectra obtained from each of these fields, the relative calcium concentrations were calculated and expressed as the ratio between the area of the calcium peak and that of the background.

RESULTS

By means of the Goldner and von Kossa procedures, previously described mineralization patterns were observed in all three cases[10,11]. Thus, completely mineralized, unmineralized and partly mineralized trabeculae were observed. In the latter areas the typical sandwich-pattern could be found in several places. When the results of the Goldner stain were compared with those of the von Kossa procedure, it seemed as if larger areas of mineralized trabeculae could be visualized by means of the former method than with the latter one. These histochemical findings indicated that a more or less continuous spectrum of mineralization could occur within one and the same trabecula, as well as between the different trabeculae.

The results, obtained from the two conventional histochemical procedures to detect calcium, could be verified by means of the EDX analysis. As illustrated in Figure 2, the relative calcium concentration of a mineralized trabecula could be about 25 times higher than that of an unmineralized one. The variation in mineralization corresponded closely to the calcium stainings. When three sites in one trabecula were measured, one highly mineralized ("I", Figure 3) and two partially mineralized in a sandwich pattern ("II", "III", Figure 3), the relative calcium concentration in the highly mineralized part of the trabecula was again found to be about 25 times higher than that of the poorly mineralized areas (Figure 4). The relative calcium concentrations within areas with a sandwiched staining pattern varied by a factor of 10. Again, the variation in mineralization was in accordance with the stainings for calcium.

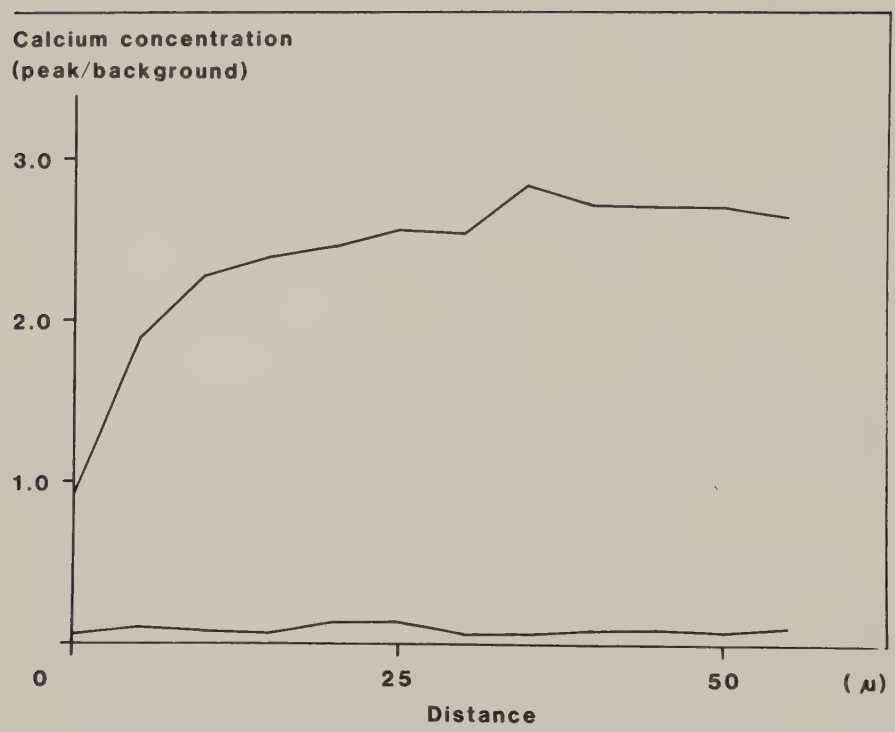

Figure 2. Peak/background ratios determined by means of EDX, in a series of about 12 consecutive measurements in one mineralized and one almost completely osteoid trabecula as judged from both the Goldner and the von Kossa stainings. The measurements were inwards from the trabecular surfaces and show that the relative calcium concentration in a mineralized trabecula is, in its central parts, about 25 times higher than in a non-mineralized osteoid trabecula.

Figure 3. Photomicrograph of a "Goldner stained" bone trabecula from one of the three cases with post-traumatic osteopenia. Osteoid appears dark and mineralized bone light (x25).

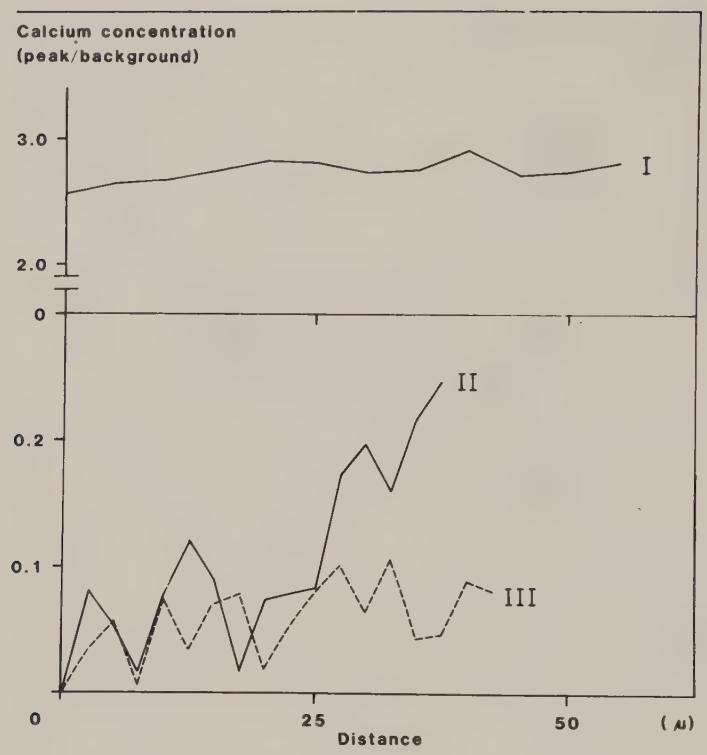

Figure 4. Peak/background ratios in the three areas of highly mineralized ("I" in Figure 3) and partially mineralized ("II", "III" in Figure 3) bone trabecula, measured at 12 - 20 fields along the lines indicated in Figure 3. The measurements were made from the trabecular surface and inwards. As shown in the upper graph ("I"), the relative calcium concentration in the highly mineralized part of the trabecula was about 10 - 25 times higher than that in the poorly mineralized areas, i.e. the two lower graphs ("II", "III"). The diagrams also show that the layered staining patterns ("II", "III") actually correspond to fairly large fluctuations in the calcium concentration in the trabecula.

Obvious sandwiched staining patterns were present in both the von Kossa and the Goldner stainings. In order to test with the electron probe whether a sensitivity difference actually exists between the two stainings, adjacent serial sections were made from trabeculae with sandwiched mineralization patterns, where the two histochemical stainings gave different results. Two such trabeculae, one from each staining, were analyzed along a line perpendicular to the sandwich pattern (Figures 5 and 6). As illustrated in the figures, the Goldner procedure seems to be about 10 times as sensitive as the von Kossa staining technique. A pronounced agreement is also demonstrated between the two stainings, von Kossa as well as Goldner, and the variation in relative calcium concentration estimated by the electron probe.

DISCUSSION

The use of fixed material for measurements of element concentrations constitutes a good reason to be cautious. There is an obvious risk of washing out substances during the fixation or dehydration procedures. Nicholson et.al.[14] have shown,though, that calcium in mineralized tissue is tightly bound to the matrix and withstands the chemical procedures well, whereas phosphorus for example is easily washed out. For this reason, and because of our aim to correlate our data with the results of histochemical stainings, we judged it correct to use fixed and dehydrated bone tissue for this investigation.

In recent studies a morphological pattern was found in trabecular bone of patients with recent fractures[10,11].Unmineralized areas and areas with layered mineralization were found much more abundantly than in any other bone disease. Basing their opinion on a micro-radiographic examination of two cases, Baud and Pouëzat[8] suggested that after fracture the bone mineral content of entire trabeculae may be decreased.Others[6,7] have found that in bone after trauma, minor parts of a trabecula can have decreased mineral content in the form of smaller mineral crystals. This concept of local bone "demineralization" is in conformity with recent findings in analogous experiments using immobilized monkeys[15].

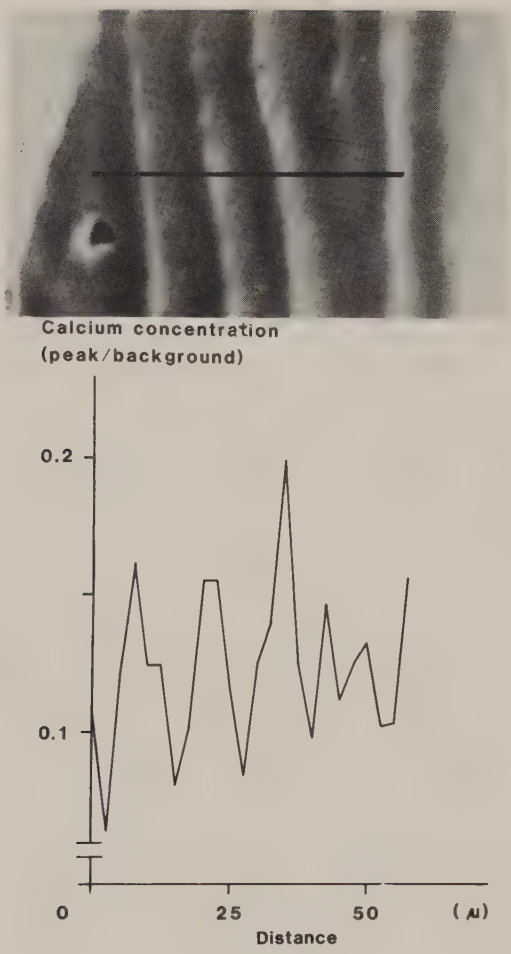

Figure 5. Photomicrograph of a "Goldner stained" section of a poorly mineralized trabecula with the sandwich pattern of mineralization such as that shown in Figure 3 ("III") The calcium-positive areas appear light. (The corresponding consecutive section stained according to von Kossa, not shown in the figure, is negative for calcium). A third, unstained, consecutive section was used for the EDX analysis of the calcium content along the line indicated in the photomicrograph. The distances are given in μm ("μ" in the graphs). The Goldner procedure gives clearly positive results for the presence of calcium in the bone trabecula at a concentration where the von Kossa fails to indicate the presence of calcium in any parts.

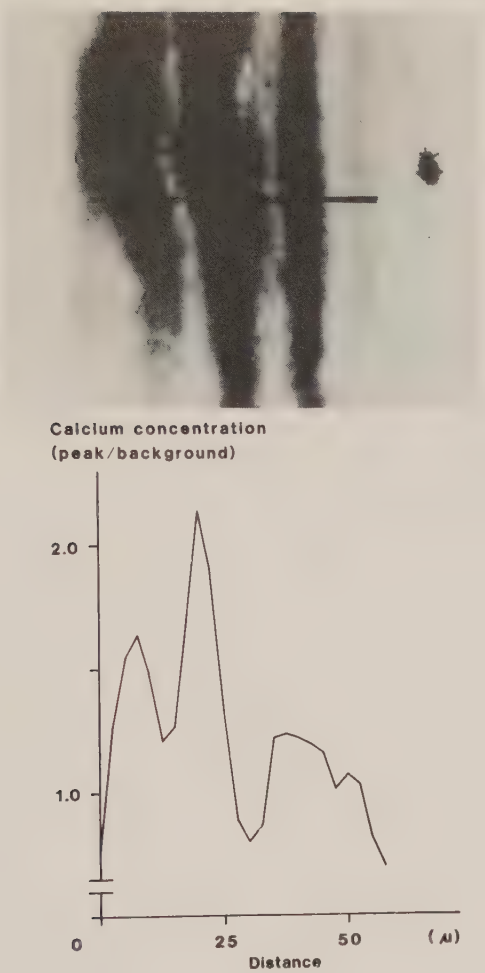

Figure 6. Photomicrograph of a "von Kossa stained" area of a more mineralized bone trabecula. The calcium-positive areas appear black. Three adjacent serial sections were analyzed histochemically by the Goldner and the von Kossa procedures as well as by means of EDX, as described in detail in Figure 5. (The corresponding "Goldner stained" section, not shown in the figure, is positive for calcium). As shown in the diagram, the von Kossa procedure here gives a positive reaction at a calcium concentration giving peak/background ratios about ten times higher than in Figure 5.

A possible explanation of these phenomena is offered by the theory of calciolysis[16,17]. However, there is evidence that post-traumatic osteopenia is a high turn-over condition with increased bone resorption and increased bone formation[11,18]. New bone may be formed at a rate that does not allow for a normal mineralization. Since the intensity in this process may vary between different parts of the bone tissue, and at different times, (there is by no means any constant distance between the sandwiched layers in different trabeculae[11]), a variation in calcium concentration is readily explained. It is possible that the most uniformly mineralized trabeculae in post-traumatic osteopenia represent the older regions of bone, and that this uniform density is due to the advent of processes of secondary mineralization described by Wergedal and Baylink[19]. Large amounts of bone tissue with a low degree of mineralization also offer an explanation of the partial reversibility of post-traumatic osteopenia in man[1,20].

The interpretation of the staining characteristics, when using histochemical stainings such as the Goldner and von Kossa techniques for example, has to date been based on rather limited knowledge of the concentrations of the substances involved. In this respect these two methods of estimating bone mineralization do not differ from other histochemical techniques. The fact that the staining characteristics of the von Kossa technique is related to the content of calcium, is shown with the EDX technique by Gardner and Hall[21]. The present study gives proof that this is also valid for the Goldner technique. We have, furthermore, clearly demonstrated that the staining techniques of von Kossa and Goldner reflect the calcium concentration level. Moreover, we have confirmed that the sensitivity for calcium differs strongly between these two routine methods. This provides a good explanation of the controversial reports on the occurrence of osteoid, not only in various pathological conditions, but also in biopsy specimens from bone of normal individuals[22].

REFERENCES

1. Nilsson, B.: Post-traumatic osteopenia. Acta orthop. scand. Suppl. 91, 1966.
2. Björk, L. and Lemperg, R.: Radiographic determination of the bone mineral content in amputation stumps. Acta Radiol. 6:575, 1967.
3. Westlin, N.E.: Loss of bone mineral after Colles' fracture. Clin. Orthop. 102:194, 1974.
4. Rieder, W.: Die akute Knochenatrofie. Dtsch. Z. Chir. 248,1936.
5. Basle, M.F., Rebel, A. and Renier, J.C.: Bone tissue in reflex sympathetic dystrophy syndrome - Sudecks atrophy: Structural and ultrastructural studies. Metab. Bone Dis. Rel. Res. 4:305, 1983.
6. Frank, R.M., Kern, P. and Fontaine, R.: Etude histopathologique au microscope électronique de l'ostéoporose post-traumatique. Pathologie-Biologie 5:2203, 1957.
7. Meyer, A.: Recherches sur l'infrastructure du tissu osseux. Faculté de Médicine de Strasbourg No. 29, Thesis, 1960.
8. Baud, C.A. and Pouëzat, J.A.: Données microradiographiques quantitatives sur le minéral osseux dans l'ostéoporose posttraumatique. Rhumatologie (Aix-les-Bains). 25:75, 1973.
9. Goldner, J.: A modification of the Masson trichrome technique for routine laboratory purpose. Amer. J. Path. 14:237, 1938.
10. Obrant,K.: Trabecular bone changes in the greater trochanter after fracture of the femoral neck. Acta orthop. scand. 55: 78, 1984.
11. Obrant,K. and Nilsson, B.: Histomorphologic changes in the tibial epiphysis after diaphyseal fractures. Clin. Orthop. 185:148, 1984.
12. Cameron, G.R.: The staining of calcium. J. Path. and Bact. 33:929, 1930.
13. Burkhardt, R.: Teknische Verbesserung und Anvendungsbereich der Histo-biopsie von Knochenmark und Knochen. Klin. Wschr. 44:326, 1966.
14. Nicholson, W.A.P., Ashton, B.A., Höhling, H.J., Quint, P., Schreiber, J., Ashton, I.K. and Boyde, A.: Electron microprobe investigations into the process of hard tissue formation. Cell.

Tiss. Res. 177:331, 1977.

15.Bunch, T.E., Young, D.R. and Niklowitz, W.Y.: Disuse osteoporosis in the monkey: Electron probe analysis of cortical bone. Proc. Amer. Soc. Bone and Mineral Res. p. 3, 1982.

16.Bohatirchuk, F.P.: Studies in bone formation and osteoid. Proc. of the first workshop on bone morphometry. University of Ottawa,Ottawa, Canada 28-31 March, p. 197, 1973.

17.Aaron, J.E.: Demineralization of bone in vivo and in vitro. Evidence for a microskeletal arrangement. Proc. of the third international workshop of bone histomorphometry. Sun Valley, May 28 - June 2. Suppl. Metabol. Bone Dis. Rel. Res., 1980.

18.Frost, H.M. (Ed.): Bone remodelling and its relationship to metabolic bone diseases. Springfield, Ill. Charles Thomas,1973.

19.Wergedal, J.E. and Baylink, D.J.: Electron microprobe measurements of bone mineralization rate in vivo. Am. J. Physiol. 222-2:345, 1974.

20.Andersson, S.M. and Nilsson, B.: Changes in bone mineral content following tibia shaft fractures. Clin. Orthop. 144:226, 1979.

21.Gardner, D.L. and Hall, T.A.: Electron-microprobe analysis of sites of silver deposition in avian bone stained by the v.Kossa technique. J. Path. 98:105, 1969.

22.Wiklund, P.: The clinical significance of unmineralized bone matrix in man. Thesis, University of Lund, 1980.

IV

ELECTRON MICROPROBE INVESTIGATION OF THE CONCENTRATION OF CALCIUM AND PHOSPHORUS IN BONE TRABECULAE IN MAN - BOTH NORMAL AND IN POST-TRAUMATIC OSTEOPENIA

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INTRODUCTION

It is known from histochemical studies that trabecular bone of a traumatized limb may be poorly mineralized (1,2). There may be areas of low mineral content even in central parts of the trabeculae (3). Histochemical techniques, however, permit only comparative estimation of the calcium content in bone trabeculae (4).

Energy dispersive X-ray microanalysis (EDX-technique, electron microprobe technique) permits the calculation of the concentration of calcium in any given part of a bone sample (5). Using this technique Bunch et al. (6) found decreased mineral content when measuring cortical bones of immobilized monkeys. In a recent study on patients who had had a fracture of the tibia diaphysis we demonstrated, also with the electron microprobe technique, large variations of the calcium content in different epiphyseal trabeculae of the same bone (7). Absolute quantification of the mineral concentration in human post-traumatic bone trabeculae has,however, not been undertaken before.

The aim of this investigation was to measure the concentration of calcium and phosphorus in central parts of human bone trabeculae after trauma, and to make a comparison with normal bone.

MATERIAL AND METHODS

With a Burkhardt drill (8) biopsy specimens, 0.5 cm in diameter and approximately 1.5 cm long, were taken from the proximal tibia epiphysis (Figure 1), in five men aged 30 ± 6 years who had had a fracture of the ipsilateral tibia diaphysis 6 - 12 months earlier. All five patients had been scheduled for secondary surgical treatment - the insertion of an intra-medullary nail - of the tibia shaft fracture because of delayed union. This operation provided an opportunity to obtain a biopsy without interfering with the healing process. In no case was the site of the biopsy closer to the fracture than 18 centimeters. Samples from the same location were obtained from five men aged 45 ± 19 years who had suffered sudden death.

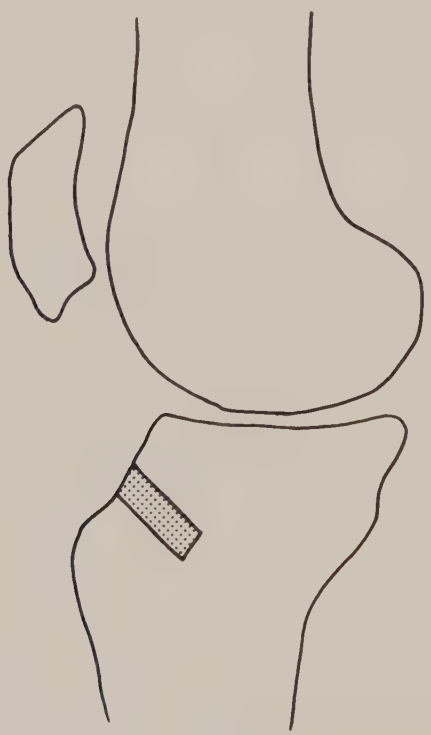

Figure 1. Sample site - a drill hole immediately proximal to the tibia tuberosity used for insertion of the intra-medullary nail.

After fixation in buffered formalin, the bone biopsy specimens were embedded in methylmethacrylate. Sections, 5 µm thick, were cut by a hard sectioning, Jung microtome and stained according to Goldner (9). Histomorphometric calculation of the osteoid volume (OV%) (10) was performed for each sample. In addition, a grind section, about 100 µm thick, was prepared from each sample. These thick sections were mounted on carbon plates, carbon coated in a vacuum evaporator and analysed in a SEM-STEM* equipped Jeol 200 CX electron microscope connected to a Link 860 energy dispersive microanalyser.

In each of the 5 + 5 cases bone trabeculae were identified in the electron microscope and 10 randomly located measurements were made (Figure 2). Care was taken to spread the mesurements as much as possible to different trabeculae within each section. All measurements were made in the SEM mode in a central part of the trabeculae. The area of each measured field was 160 μm^2. In no case were the trabecular edges in the section closer than 40 µm to the measured field and no trabeculae thinner than 90 µm were examined. The concentration of calcium and phosphorus was calculated from the spectrum of each field by means of a computer program.**

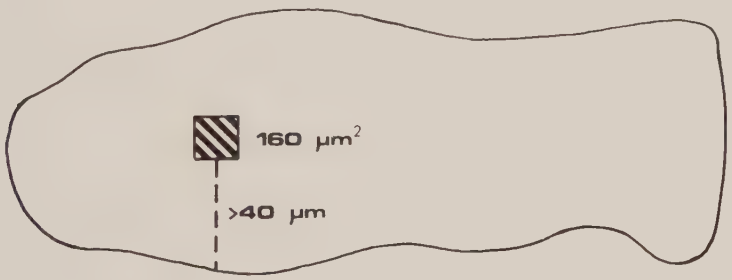

Figure 2. Measuring site within a trabecula. A square area was located centrally in the trabecula, never less than 40 µm from the edge.

* Scanning electron microscopy - scanning transmission electron microscopy

** ZAF peak-to-background program, Link Systems Ltd.

RESULTS

The OV% was 8.4 ± 7.8 (mean ± 1 SD) in the post-traumatic patients as compared with 0.8 ± 1.1 in the control cases. The concentration of calcium in the central parts of the trabeculae also differed between the two groups ($p < 0.01$ Mann-Whitney test) (Figure 3). The post-traumatic patients had a decreased concentration as compared with the controls, 29 ± 10 and 45 ± 1 wt%, respectively. There was more scatter in the post-fracture data ($p < 0.001$, F-test). The corresponding concentrations of phosphorus were 12 ± 5 and 21 ± 1 wt%, respectively. The mean ratio of calcium to phosphorus concentrations was 2.5 ± 0.3 in the post-traumatic group and 2.1 ± 0.01 in the controls, a significant difference ($p < 0.01$) and also a significantly wider distribution of the values ($p < 0.001$). There was no correlation between OV% and calcium content.

DISCUSSION

The electron microprobe technique for quantitative assays of single elements in tissue sections is today a well established method (11). The results of the present study show that it is well suited also for mineralized tissues. Compared with ashing studies the electron microprobe technique has the advantage of permitting measurements in anatomically defined parts of a bone sample.

Nicholson et al. (12) have shown that in biological material calcium is tightly boundto the matrix and not, as in the case of phosphorus, partly washed out during the process of preparation. In our measurements the proportion of calcium to phosphorus was almost constant in the control cases, whereas in the post-traumatic group, in addition to a slightly increased ratio, there was an increased scatter. It is not clear why the calcium/phosphorus ratio is increased in the post-traumatic cases. It is hard to believe that the wash out phenomenon described by Nicholson et al. (12) would be enhanced at lower concentrations of phosphorus. Unless the decreased absolute concentration of calcium *per se* enhances the washing out of phosphorus, the increased

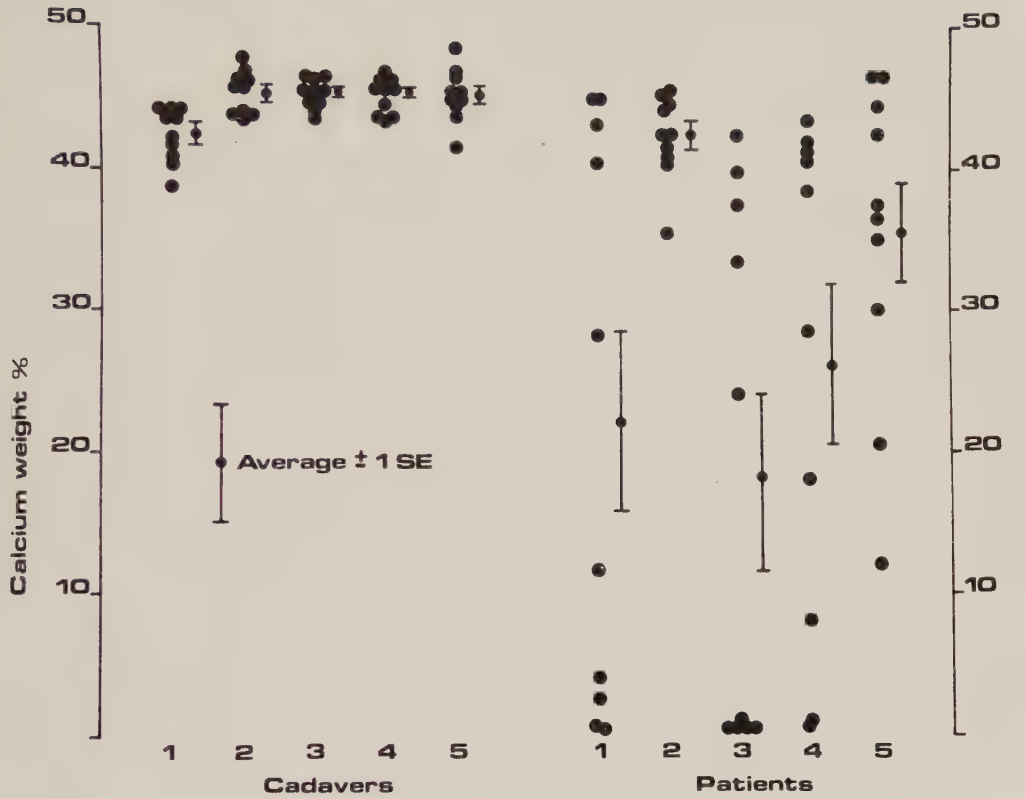

Figure 3. A diagram showing the amount of calcium, in weight per cent, as found in ten electron microprobe measurements for each of 5 patients with post-traumatic osteopenia and in 5 cadaver controls.

calcium to phosphorus ratio could be real in the central parts of bone trabeculae after trauma. Nevertheless, the phosphorus data of the present study are uncertain.

There was no correlation between osteoid and mineralization. However, the cases are few and the changes specific for post-traumatic osteopenia are not evenly distributed throughout the trabecular bone (3).

SUMMARY

With electron probe microanalysis the concentration of calcium and phosphorus was determined in central parts of tibia epiphyseal bone. Trabeculae from five men who had had a fracture of the ipsilateral tibia diaphysis were analysed. The results were compared with those of five men who had suffered sudden death.

There was an even distribution of calcium in the control cases - the mean value was 45 $\pm$ 1 weight %. In the post-traumatic group of patients there was a lower mean and a greater scatter - 29 $\pm$ 10 wt%. Also, the concentration of phosphorus was lower in the post-traumatic cases as compared with the cadavers.

REFERENCES

1. Rieder W (1936) Die akute Knochenatrophie. Dtsch Z Chir 248:269-331.
2. Obrant K (1984) Trabecular bone changes in the greater trochanter after fracture of the femoral neck. Acta Orthop Scand 55:78-82.
3. Obrant K, Nilsson B (1984) Histomorphologic changes in the tibial epiphysis after diaphyseal fracture. Clin Orthop 185:148-153.
4. Baron R, Vignery A, Neff L, Silverglate A, Santa Maria A (1983) Processing of undecalcified bone specimens for bone histomorphometry. In: Bone Histomorphometry: Techniques and interpretation. Ed: Recker, R. Publ. CRC Press, Florida, pp 13-35.

5. Wergedal JE, Baylink DJ (1974) Electron microprobe measurements of bone mineralization rate in vivo. Am J Phys 226:345-352.
6. Bunch TE, Young DR, Niklowitz WY (1982) Disuse osteoporosis in the monkey: Electron probe analysis of cortical bone. Proc Amer Soc Bone and Mineral Res, p 3.
7. Obrant K, Odselius R (1984) Electron microprobe analysis and histochemical examination of the calcium distribution in bone trabeculae in man. A methodological study, using biopsy specimens from post-traumatic osteopenia.Submitted for publication.
8. Burkhardt R (1966) Technische Verbesserung und Anwendungsbereich der Histo-Biopsie von Knochenmark und Knochen. Klin Wschr 44:326-334.
9. Goldner J (1938) A modification of the Masson trichrome technique for routine laboratory purpose. Amer J Path 14:237-243.
10. Melsen F, Mosekilde L (1981) The role of bone biopsy in the diagnosis of metabolic bone disease. Orthop Clin North Am 12:571-602.
11. Hayat MA (Ed) (1980) X-ray Microanalysis in Biology. University Press, Baltimore.
12. Nicholson WAP, Ashton BA, Höhling HJ, Quint P, Schreiber J, Ashton IK, Boyde A (1977) Electron microprobe investigations into the process of hard tissue formation. Cell Tiss Res 177:331-345.

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POST-FRACTURE CHANGES OF THE FEMUR CORTEX

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Roentgenograms of the femora were obtained in 21 patients who on average 10 years earlier had sustained a fracture of the shaft of one tibia. On the fracture side a slightly uneven "spotted" appearance could be detected in most patients. Also, the marrow canal was about 10 per cent wider in the femora of the fractured limb – this widening was unrelated to the time factor, indicating a permanent loss of endosteal bone.

Key words: cortical bone; fracture; osteoporosis.

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A fracture causes a loss of bone, not only from the fracture site but also from other parts of the fractured limb. This has been demonstrated in animals and in man (Bauer 1954, Nilsson 1966, Westlin 1974, Andersson & Nilsson 1979). All measurements in man have been conducted in the trabecular bone near the ends of the fractured bone or in the neighbouring bones. However, Wendeberg (1961) was able to demonstrate that not only the fractured tibia and the bone tissue of the knee joint, but also the shaft of the femur is participating in the high mineral turnover process and Garn et al. (1963) suggested the cortical thickness of the femoral shaft to be a good measuring site for evaluation of bone mass.

The objective of this investigation was to study the long-term effects of fracture on the femur cortex.

MATERIAL AND METHODS

Included in the study were 15 men and 6 women, age 49 ± 13 years, who on average 10 years earlier (range 2–17) had sustained a fracture of the shaft of the tibia. The age at the time of the fracture was 39 ± 14. The subjects were selected from a larger series using the time of immobilization as the only selection criterion – it was a condition that the plaster immobilization should have exceeded the average time (4.5 months). Immobilization ranged from 4.5 to 40 months (9 ± 7) and all fractures had subsequently healed.

The subjects all had a roentgen examination including only the antero-posterior view of the mid-portion of both femora in a standard rotation position with focus in the mid-line. The films were measured and evaluated by an investigator who was not aware which leg had been fractured with regard to the following variables.

Endosteal and periosteal width. The narrowest part of the marrow canal was identified. Also, measuring points were found 2 and 4 cm proximal and distal to this point – altogether 10 sites for each femur (Figure 1). The total (periosteal) thickness of the femur and the cortical thickness were measured on the same sites. The marrow width was calculated by subtraction and the ratio marrow width/total width was calculated. The average was calculated for each bone and the injured and uninjured sides were compared using the *t*-test for paired observations.

Endosteal irregularities. The subjective impression of the shape of the bone lining of the marrow cavity.

Density irregularities. Possible signs of "spottedness" of the cortical bone projected over the marrow cavity.

Also, for the two subjective estimates, the films were mixed and re-mixed.

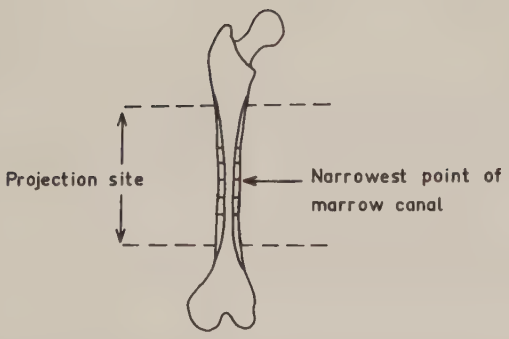

Figure 1. Measuring site.

RESULTS

The total (periosteal) width of the femora did not differ between the injured and the uninjured sides. However, the marrow canal was nearly 10 per cent wider in the fractured limb (Table 1).

The duration of plaster fixation and the time elapsing between the fracture and the follow-up measurement were not correlated* with the degree of widening of the marrow canal, nor was this variable influenced by the age at the time of fracture.

Attempts to identify the fractured limb from the film of the mid-portion of the femur by the shape of the endosteal cortex marrow border failed completely but in the image of the femur overlying the marrow cavity, the structure appeared slightly more "spotted" on the fracture side in 20 of the subjects.

* Linear correlation – marrow/total width ratio as the independent variable.

DISCUSSION

In an earlier study of roentgen findings in trabecular bone after tibia shaft fracture, the image appeared, with few exceptions, normal after 2 years (Nilsson 1966). In the present study there were subtle changes in the femoral cortex even after that time. The difference in marrow width which in the fractured limbs was increased by about 10 per cent indicates a long-lasting or possibly irreversible loss of endosteal bone. There was no tendency to restoration of bone during the time period covered by this study. Restoration, if any, must have taken place during the very first years after the injury. A loss of this magnitude should increase the risk of fracture unless the tubular shape of the femur is sufficiently regular, particularly if the loss is not uniformly distributed.

The findings suggest that not only the trabecular but also the cortical bone of an injured limb, as proposed by Wendeberg (1961), participates in the post-traumatic changes of bone resorption and formation leaving long-lasting – perhaps lifelong – roentgen-morphometric evidence of a net loss.

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REFERENCES

Andersson, S. & Nilsson, B. E. (1979) Changes in bone mineral content following tibia shaft fractures. *Clin. Orthop.* **144**, 226–229.

Table 1. Total (periosteal) width in mm and the marrow width/total width ratio

	N	Uninjured Av±SD	Injured Av±SD	Difference Av±SD
Total width, mm	21	33.6±3.7	33.6±3.8	0
Marrow/total	21	0.391±0.036	0.416±0.047	0.025±0.006 $p<0.001$

Bauer, G. C. H. (1954) Rate of bone-salt formation in a healing fracture determined in rats by means of radiocalcium. *Acta Orthop. Scand.* **23**, 169.

Bohr, H. (1955) Studies on fracture healing. *J. Bone Joint Surg.* **37**-A, 327.

Garn, S. M., Rohmann, C. G. & Nolan, P. (1963) The developmental nature of bone changes during aging. In: (Ed. Birren, J. E.) *Relations of developing and aging.* Charles C. Thomas, Springfield, Ill.

Nilsson, B. E. (1966) Post-traumatic osteopenia. A quantitative study of the bone mineral mass in the femur following fracture of the tibia in man using Americium-241 as a photon source. *Acta Orthop. Scand.* Suppl. 91.

Wendeberg, B. (1961) Mineral metabolism of fractures of the tibia in man studied with external counting of Sr^{85}. *Acta Orthop. Scand.* Suppl. 52.

Westlin, N. E. (1974) *Bone mineral in Colles fracture.* Dissertation, Lund University.

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